REMARKS

Claims 16, 19 -25 and 30-31 are presently pending. Claims 30-31 were previously withdrawn. New claims 32-36 are submitted herewith. A Request for Continued Examination is submitted herewith in lieu of an Appeal Brief. No new matter has been introduced by this amendment.

The Full Scope of Claim 16 and Claims Dependent Thereon is Enabled.

The only remaining rejection of claims 16, and 19-25 is under 35 U.S.C. § 112, first paragraph for alleged lack of enablement. Applicants' representative had a brief telephonic conference with Examiner Collins regarding the claims on August 4, 2005. Specifically, the rejection of claim 16 was discussed. The Examiner explained that the nature of the rejection was due to the alleged unpredictability of the effect of overexpressing *any* gene on the production of vanillin, and that undue experimentation would be required

Applicants respectfully traverse the rejection. The Examiner indicated that the rejection is not predicated on the scope of the phrase "comprising genetically engineering the *Vanilla planifolia* to overproduce an enzyme. . ." Applicants note that the Office Action has not presented any objective evidence of record that overexpressing a rate-limiting enzyme would not be understood by the skilled artisan as a rational approach for improving metabolite production in plants, and one which would in fact be successful. Applicants submit herewith references related to improving the production of metabolites by such techniques. It is generally understood in the art of plant metabolite production that carbon may be channeled or shunted into alternative or desired pathways by genetic manipulation. The references collectively teach that not only was it known in the art, before the time of filing, that for pathways at least as complex as that for vanillin, that the production of specific metabolites could be altered by such methods, it was even possible to increase the production of vanillin and related compounds by such methods (see e.g. Seibert *et al. Plant Physiol.* 112:811-819 (1996) and also Rasmussen and Dixon, *Plant Cell* 11: 1537-1551 (1999) (note Table 1).

Applicants respectfully request reconsideration in view of the foregoing. Applicants have fully enabled the skilled artisan to make and use the invention with at most ***routine***

experimentation. By identifying a rate-limiting step in the pathway to vanillin production, and providing the amino acid sequence of an enzyme catalyzing the rate-limiting reaction, Applicants, have enabled the skilled artisan to make *Vanilla planifolia* cells or plants overproducing the chain shortening enzyme (having the amino acid sequence of SEQ ID NO:2) by what are clearly now routine genetic engineering methods, with an expectation of success. The most that is required is routine screening for those cells or plants having improved vanillin production. Since measurement of vanillin is routine, there is no objective reason to consider that undue experimentation would be required to make and use the invention as taught and claimed.

The specification teaches the skilled artisan how to make and use the claimed invention; nothing more is required by the statute. At most, routine experimentation would be required. Applicants accordingly request withdrawal of the rejection under 35 U.S.C. § 112, first paragraph.

Conclusion

It is respectfully submitted that this reply is fully responsive to any outstanding Office Action. It is further submitted that all claims are in condition for allowance. An early and favorable Notice to that end is earnestly solicited. The Examiner is invited to contact the Applicant's undersigned representative telephonically to resolve any outstanding issues prior to allowance. Applicants' representative may be reached during normal business hours at 215-557-5986.

Respectfully submitted,

Date: September 6, 2005

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Rerouting the Plant Phenylpropanoid Pathway by Expression of a Novel Bacterial Enoyl-CoA Hydratase/Lyase Enzyme Function

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The gene for a bacterial enoyl-CoA hydratase (crotonase) homolog (HCHL) previously shown to convert 4-coumaroyl-CoA, caffeoyl-CoA, and feruloyl-CoA to the corresponding hydroxybenzaldehydes in vitro provided an opportunity to subvert the plant phenylpropanoid pathway and channel carbon flux through 4-hydroxybenzaldehyde and the important flavor compound 4-hydroxy-3-methoxybenzaldehyde (vanillin). Expression of the *Pseudomonas fluorescens* AN103 HCHL gene in two generations of tobacco plants caused the development of phenotypic abnormalities, including stunting, interveinal chlorosis and senescence, curled leaf margins, low pollen production, and male sterility. In second generation progeny, the phenotype segregated with the transgene and transgenic siblings exhibited orange/red coloration of the vascular ring, distorted cells in the xylem and phloem bundles, and lignin modification/reduction. There was depletion of the principal phenolics concomitant with massive accumulation of novel metabolites, including the glucosides and glucose esters of 4-hydroxybenzoic acid and vanillic acid and the glucosides of 4-hydroxybenzyl alcohol and vanillyl alcohol. HCHL plants exhibited increased accumulation of transcripts for phenylalanine ammonia-lyase, cinnamate-4-hydroxylase, and 4-coumarate:CoA ligase, whereas β-1,3-glucanase was suppressed. This study, exploiting the ability of a bacterial gene to divert plant secondary metabolism, provides insight into how plants modify inappropriately accumulated metabolites and reveals the consequences of depleting the major phenolic pools.

INTRODUCTION

The plant phenylpropanoid pathway is responsible for the synthesis of a wide variety of secondary metabolic compounds, including lignins, salicylates, coumarins, hydroxycinnamic amides, flavonoid phytoalexins, pigments, UV light protectants, and antioxidants (Dixon and Paiva, 1995). In addition to their roles in the structure and protection of the plant, phenylpropanoids have an important effect on plant qualities such as texture, flavor, color, and processing characteristics. Molecular engineering provides a tool to determine the complex biochemical pathways involved in the synthesis and regulation of phenylpropanoids and to manipulate pathways to increase or initiate the production of economically desirable traits or compounds (Dixon et al., 1996). Expression of plant genes in heterologous plant systems can lead to improved or novel synthesis of valuable compounds (Yun et al., 1992) and improved disease resistance (Hain et al., 1993). The overexpression or downregulation of enzymes involved in phenylpropanoid and lignin biosynthesis has demonstrated that it is possible to alter the content and properties of lignin and its associated phenolics (reviewed in Boudet, 1998). This finding has important implications for the manipulation of plant quality with respect to pulping, forage digestibility, texture, and defense responses (Campbell and Sederoff, 1996). The expression of bacterial genes in transgenic plants also has proven to be effective, both in introducing new pathways to increase the accumulation of desired compounds (Fecker et al., 1993; Siebert et al., 1996) and in facilitating understanding of the plant defense response (Delaney et al., 1994).

A novel bacterial enzyme was isolated previously from a *Pseudomonas fluorescens* strain (AN103) selected on the basis of its ability to metabolize ferulic acid via vanillin (Gasson et al., 1998; Narbad and Gasson, 1998). The bacterial enzyme, named 4-hydroxycinnamoyl-CoA hydratase/lyase (HCHL), is an enoyl-CoA hydratase with an aldolase function; in vitro, it can convert feruloyl-CoA into equimolar quantities of vanillin (4-hydroxy-3-methoxybenzaldehyde) and acetyl-CoA (Figure 1). Vanillin is the major flavor component of vanilla, one of the most important flavoring substances. The HCHL enzyme also converts 4-coumaroyl-CoA and caffeoyl-CoA in vitro to the corresponding hydroxybenzaldehydes (Mitra et

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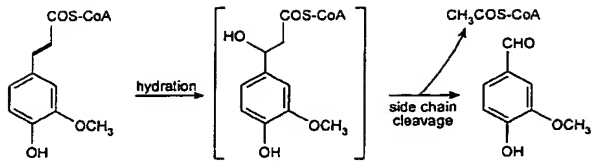

Figure 1. Conversion of Feruloyl-CoA to Vanillin by the HCHL Enzyme.

HCHL performs the hydration and cleavage of feruloyl-CoA to produce vanillin and acetyl-CoA in vitro. The enzyme also is able to reproduce feruloyl-CoA, in addition to vanillin and acetyl-CoA, from the artificial substrate and putative reaction intermediate 4-hydroxy-3-methoxyphenyl-β-hydroxypropionyl-CoA (HMPHP-CoA) (Gasson et al., 1998).

al., 1999), one of which (4-hydroxybenzaldehyde) is a minor but important component of vanilla flavor. The enzyme does not act on sinapoyl-CoA. Expression of the Pseudomonas HCHL gene in plants has the potential to divert phenylpropanoid metabolism to produce novel metabolites based on hydroxybenzaldehyde derivatives. We have expressed the HCHL gene in two generations of tobacco and analyzed the phenotypic, biochemical, and molecular consequences.

RESULTS

Generation of Tobacco Plants Expressing the Pseudomonas HCHL Gene

The wild-type form of the Pseudomonas HCHL gene and a modified form altered by one nucleotide to a more typical eukaryotic translation initiation context (atga to atgg; Kozak, 1986) were fused to the constitutive *cauliflower mosaic virus* (*CaMV*) 35S RNA promoter containing duplicated enhancer sequences. To express the bacterial gene in plants, we subcloned the expression cassettes into the *Agrobacterium tumefaciens* binary vector pBin19 and introduced them into tobacco by Agrobacterium-mediated leaf disc transformation. A total of 20 primary transformants were regenerated from leaf discs and eventually transferred to soil. The presence of the transgene in the regenerated plants was confirmed by genomic gel blot analysis (results not shown), and steady state mRNA accumulation was analyzed by hybridization of RNA gel blots with the HCHL gene fragment. Thirteen of the transformants expressed the transgene at detectable but varying levels (Figure 2); on longer exposures, all transformed plants expressed the transgene (results not shown). Three of these plants contained the wild-type form of the HCHL gene, whereas the rest contained the modified form.

Phenotypes of HCHL Primary Transformants and Their Progeny

Of the 13 independent primary transformants expressing detectable levels of HCHL mRNA, three plants with the highest accumulation of the transcript (plants 201, 27, and 39) exhibited severely abnormal phenotypes from an early stage upon transfer to soil in the greenhouse. The abnormal phenotype was characterized by the development of interveinal leaf chlorosis and thin, curled leaf margins (Figure 3A). Leaf chlorosis began as paler green areas between veins that appeared relatively sunken and shiny compared with adjacent tissue. As the leaves aged, the lighter green areas became yellow; this was followed by a brown, senescent-like appearance, with the senescent-like tissue spreading out between the veins, which remained green (Figure 3B). The senescent-like areas of the transgenic leaves were paler than was the natural senescence of the old leaves of wild-type plants. During flowering, the three plants exhibiting leaf chlorosis produced little pollen and were male sterile. Three other plants (transformants 3, 6, and 22) did not develop interveinal leaf chlorosis but did produce first flowers with very reduced pollen content that failed to set seed after self-fertilization, although later flowers were male fertile. No obvious phenotypic abnormalities were detected in any of the other HCHL-expressing plants.

Four of the primary transformants were selected for further study: three plants exhibiting the interveinal leaf chlorosis phenotype (plants 201, 39, and 27) and a fourth plant that expressed the HCHL transgene but did not exhibit phenotypic abnormalities (plant 13). Only plant 201 expressed the unmodified form of the transgene. To analyze segregating progeny of the second generation, we pollinated the three male-sterile transformants with wild-type pollen, whereas

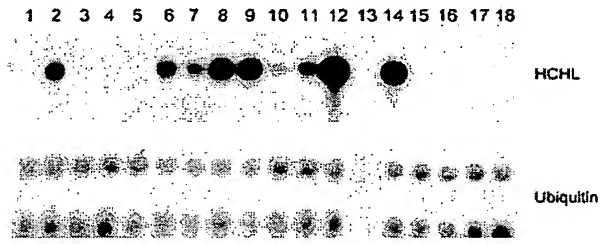

Figure 2. Steady State mRNA Levels of the HCHL Transcript in Independent Primary Transformants.

Total RNA from young leaves of primary transformants was hybridized to the HCHL gene fragment. Lanes 1 to 12, plants containing the pmHCHL construct: plants 3, 6, 7, 9, 11, 13, 21, 22, 27, 34, 37, and 39; lane 13, no sample; lanes 14 to 16, plants containing the pHCHL construct: plants 201, 210, and 224; lanes 17 and 18, control plants transformed with the empty pBin19 vector. The same blot was stripped and hybridized to a *Datura stramonium* ubiquitin probe.

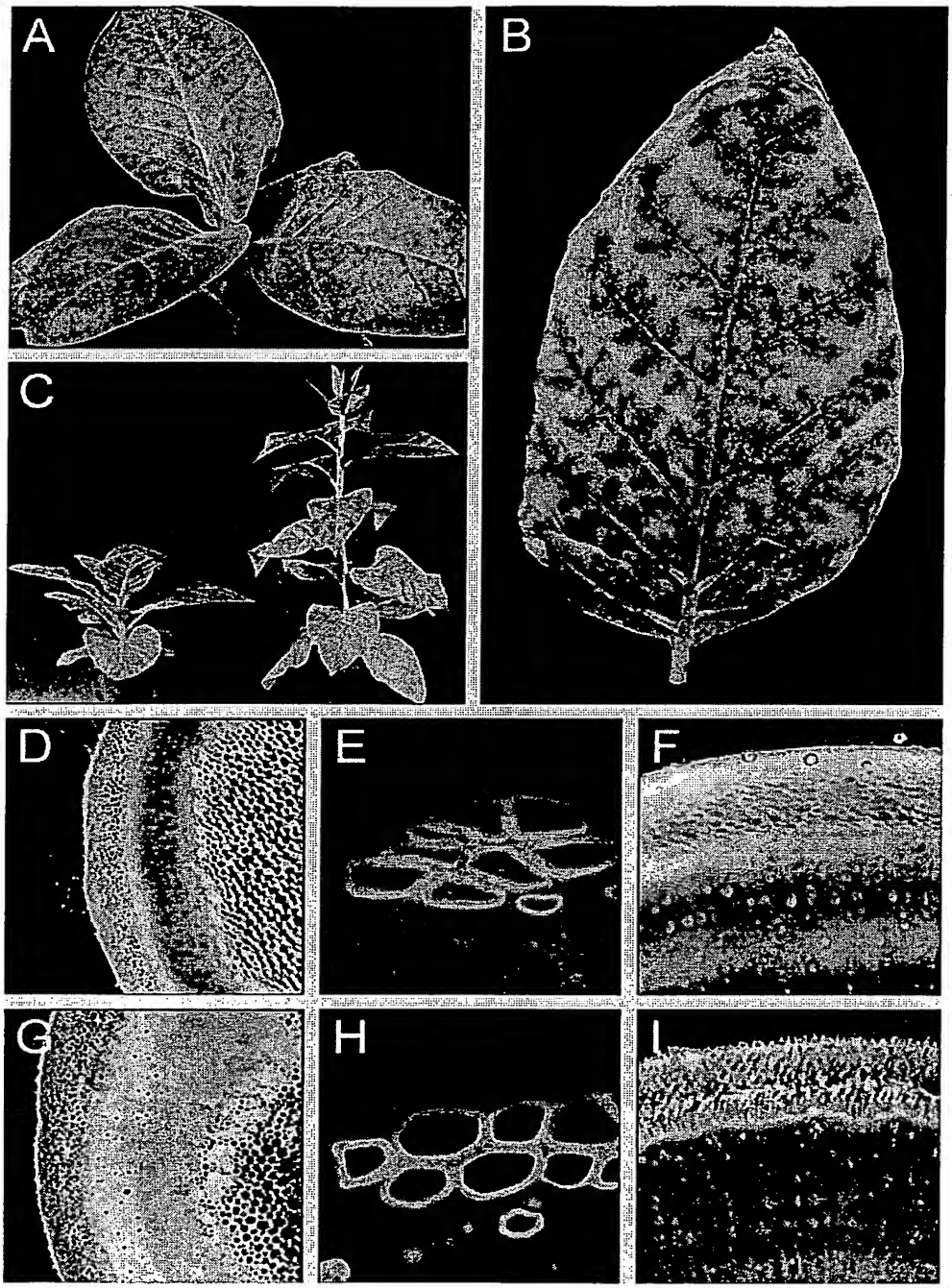

Figure 3. Morphology and Microscopic Analysis of Transgenic Plants.

(A) Primary transformant 201 at 22 days after transfer to the greenhouse.

(B) Detached leaf of primary transformant 201 at 74 days after transfer (after flowering).

(C) to **(I)** Comparisons between transgenic and syngenic progeny of line 201. **(C)** shows sibling plants of line 201 at 90 days after sowing, demonstrating delayed elongation of stem internodes in transgenic plants (left). Unstained stem sections showing an orange/red coloration of the vascular ring in transgenic plants **(D)** compared with the yellow color observed in syngenic plants **(G)** and distortion of phloem fibers in the transgenic stem **(E)** compared with the syngenic plants **(H)** were viewed under UV light fluorescence. Phloroglucinol staining of vascular tissue in transgenic stems **(F)** was reduced compared with that of the syngenic samples **(I)**.

primary transformant 13 was self-fertilized. Resulting seed were sown directly onto soil, and the transgenic status of the progeny plants was assessed by gel blot analysis of leaf RNA (see below). Progeny from all four lines clearly segregated into transgenic and syngenic siblings, with a complete correlation between HCHL expression and abnormal phenotypes. It was not possible to easily categorize transgenic siblings into homozygous or heterozygous phenotypes. Transgenic progeny from all four lines exhibited the interveinal leaf chlorosis phenotype when young, which in the case of line 13 indicated penetration of the abnormal phenotype only in the second generation.

From an early stage, the transgenic progeny of line 201 developed thin, curled leaf margins and leaf tip chlorosis before the development of interveinal chlorosis. Transgenic progeny of this line were markedly stunted compared with the syngenic siblings (Figure 3C). Eventually, the internodes of the transgenic line 201 progeny elongated, so that flowering occurred at approximately the same height as in the syngenic siblings but with a delay of 2 to 3 weeks. Initial flowers of the transgenic line 201 plants were male sterile, but later flowers were male fertile. None of the transgenic progeny from the other three lines exhibited stunting or flowering delay, and the interveinal leaf chlorosis was much less severe than in line 201 plants. Flowers of these other lines became male fertile soon after the beginning of flowering, with only a few flowers failing to develop seed capsules after self-pollination. In all four lines, the petals of the transgenic plants were noticeably paler that those of the syngenic siblings.

Examination of detached leaves of line 201 segregating progeny under UV light (365 nm) revealed a distinct difference in fluorescence between transgenic and syngenic siblings (results not shown). In syngenic leaves, the red-orange autofluorescence emitted by chloroplasts in response to excitation by UV light was shielded by phenolic compounds, so that the leaves appeared dark green/blue (Chapple et al., 1992; Tamagnone et al., 1998). In contrast, transgenic leaves appeared red-orange under UV light, suggesting depletion of the phenolic content.

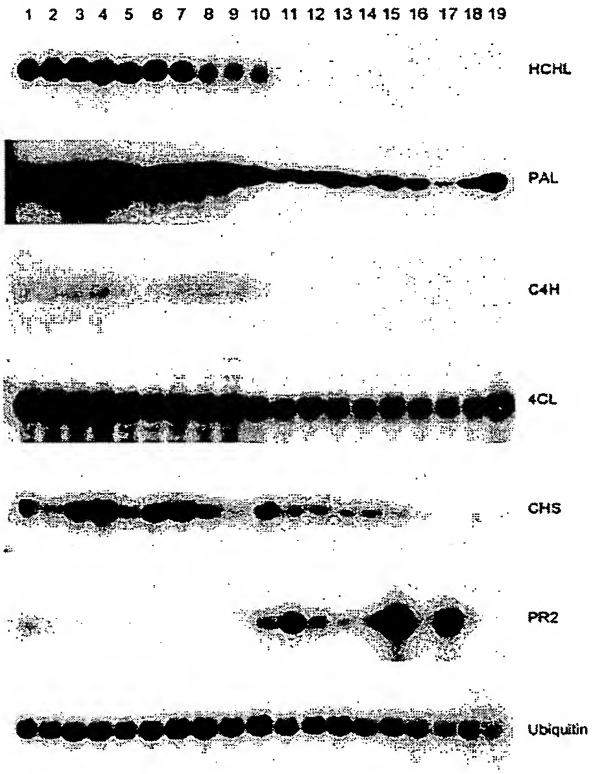

Figure 4. Steady State mRNA Levels of the HCHL Message and Endogenous Plant Genes in Progeny of Line 201.

Lanes 1 to 10, transgenic; lanes 11 to 19, syngenic.

Microscopic Examination of Second Generation Segregating Progeny

Hand-cut sections of stem taken from nodes 10 and 12 of transgenic and syngenic plants of line 201 during late flowering were examined unstained using light microscopy. The vascular ring in stems of transgenic plants had developed a red-orange coloring compared with the yellow of the syngenic siblings (Figures 3D and 3G). At higher magnification, the xylem vessels in transgenic and syngenic plant stems appeared to have a similar cell wall thickness, but some distortion and buckling was apparent in both xylem vessels and phloem bundle cells of transgenic plants (Figures 3E and 3H). The effect of HCHL expression on lignin content was examined by microscopic analysis of phloroglucinol-stained stem sections. Deep cherry-red coloring of syngenic plant stem sections was reduced in intensity and extent in the transgenic siblings (Figures 3F and 3I), indicating a change in the quantity and/or quality of lignin.

Effect of the HCHL Transgene on Expression of Genes of the Phenylpropanoid Pathway

A total of 79 second generation progeny plants of lines 201, 39, 27, and 13 were examined by RNA gel blot analysis, confirming 100% linkage of HCHL expression with the development of phenotypic abnormalities. To assess the effect of transgene expression on the mRNA accumulation of the phenylpropanoid pathway biosynthetic enzymes, probes for phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), and 4-coumarate:CoA ligase (4CL) as well as chalcone synthase (CHS) and the pathogenesis-related protein β-1,3-glucanase (PR2) were used to hybridize to total

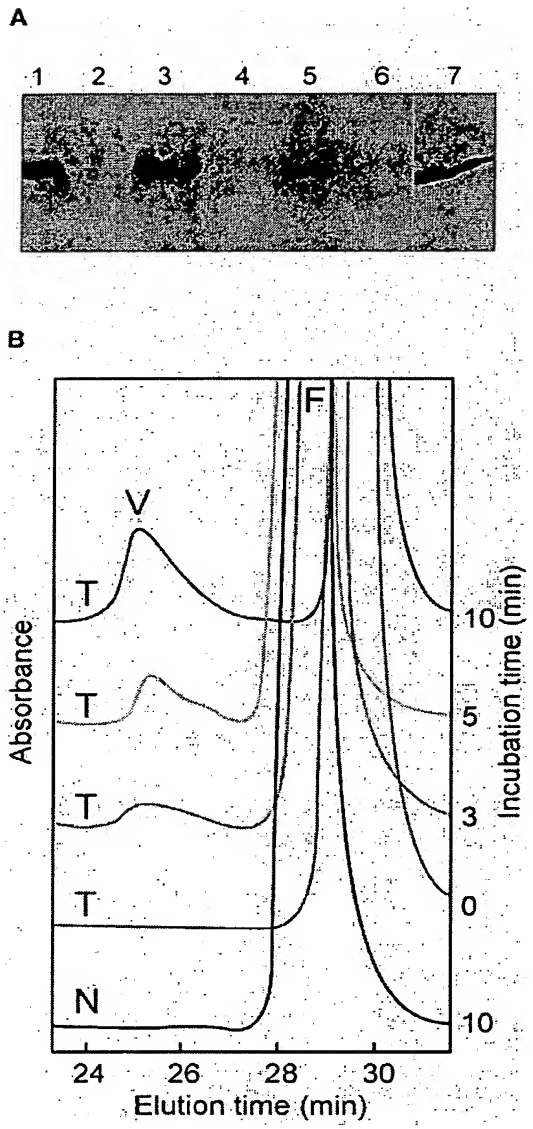

Figure 5. HCHL Enzyme Synthesis in Young Leaves of Transgenic Progeny and Activity in Vitro.

(A) Protein gel blot analysis of total protein extracts from lines 201 and 27. Lane 1, transgenic, line 201, set 1; lane 2, syngenic, line 201, set 1; lane 3, transgenic, line 201, set 2; lane 4, syngenic, line 201, set 2; lane 5, transgenic, line 27; lane 6, syngenic, line 27; lane 7, total protein extract from Pseudomonas strain AN103. Line 201 was sown on two separate occasions (sets 1 and 2).

(B) In vitro production of vanillin (V) by the incubation of 10 μL of cell-free extracts from a transgenic offspring (T) of line 201 with feruloyl-CoA (F) at 30°C for 0, 3, 5, and 10 min. Incubation with extracts from a syngenic plant (N) showed no accumulation of vanillin at any time. HPLC analysis was viewed at 310 nm.

leaf RNA from segregating progeny of line 201 (Figure 4). Leaves were analyzed before the development of the chlorosis phenotype. Steady state mRNA levels for the phenylpropanoid pathway enzymes PAL, C4H, and 4CL were clearly increased, and CHS was generally increased in the transgenic progeny, whereas the stress-associated PR2 gene was generally suppressed. We do not know whether these changes were a direct consequence of changes in phenylpropanoid metabolite levels or an indirect response to the cellular perturbation occurring in the transgenic leaves.

HCHL Enzyme Synthesis and Activity in Transgenic Progeny

Synthesis of the HCHL protein in transgenic leaves was confirmed by protein gel blotting and detection with a polyclonal antibody (Figure 5A). The antibody did not recognize any proteins in the syngenic leaf samples. One protein band of ~31 kD was detected in extracts from transgenic plants and was similar in size to the protein found in the original Pseudomonas strain that was the source of the HCHL gene (Gasson et al., 1998).

HCHL enzyme activity was measured using feruloyl-CoA as a substrate with subsequent detection of vanillin production by HPLC (Figure 5B, Table 1). The identification of vanillin was confirmed by gas chromatography–mass spectrometry (results not shown). Extracts from young leaves of second generation plants were made from the same liquid nitrogen–frozen ground powder used for RNA extraction and biochemical analysis. In all cases, in vitro vanillin production and hence HCHL activity were absent in syngenic plant extracts, whereas all transgenic plant extracts produced vanillin in varying quantities. Both the control and transgenic plant extracts contained unidentified activity that depleted feruloyl-CoA to some extent, producing an unidentified peak running at 11 min, so the incubation times for the HCHL assays were reduced to 10 min. Under these conditions, exhaustion of the substrate was prevented and the enzyme assay was linear. The HCHL enzyme activities correlated well with the level of HCHL mRNA, with line 13 progeny having more than twofold less activity than lines 201 and 39. Line 27 was an exception in that its HCHL activity was lower than predicted from the relative HCHL mRNA accumulation in the parent plant.

Biochemical Changes in HCHL Second Generation Plants

To determine changes in the level of soluble phenolics in HCHL-expressing transgenic progeny, we used HPLC with UV light detection at 220 and 255 nm (Figure 6) to analyze methanol extracts of leaves, unopened flower buds, and seed capsules. Marked differences in phenolic content were observed in young leaves of transgenic and syngenic plants

Table 1. In Vitro HCHL Enzyme Activities in Young Leaves from Transgenic Progeny

Plant Line[a]	No. Plants Sampled (*n*)	Mean Enzyme Activity ± SE (pmol vanillin per sec/μg protein)
201, set 1, T	4	0.152 ± 0.019
201, set 1, N	4	ND[b]
201, set 2, T	10	0.144 ± 0.019
201, set 2, N	9	ND
27, T	12	0.070 ± 0.009
27, N	7	ND
39, T	10	0.138 ± 0.010
39, N	1	ND
13, T	5	0.061 ± 0.014
13, N	5	ND
201, set 1, F, T	5	0.221 ± 0.051
201, set 1, F, N	3	ND

[a] F, taken during flowering; T, transgenic; N, syngenic. Line 201 was sown on two separate occasions (set 1 and set 2).
[b] ND, not detected.

before flowering. In young leaves of syngenic plants, the major phenolic peaks corresponded to chlorogenic acid (5-caffeoylquinic acid), its isomers (4-caffeoylquinic acid and 3-caffeoylquinic acid), and the major flavonoid rutin (quercetin 3-β-D-rutinoside). In young leaves of transgenic plants, these phenolics were depleted severely, although the results for rutin were less consistent. The relative depletion of these phenolics was more pronounced in the late leaves of flowering plants of line 201, with an average 93% reduction in chlorogenic acid in the transgenic progeny (Table 2). All of the major phenolics in syngenic plants were present at higher levels in the later leaves of flowering plants compared with its presence in young leaves before flowering. Depletion of these major phenolics was less pronounced in lines 27 and 13, which also exhibited lower HCHL enzyme activities.

The rerouting of the phenylpropanoid pathway away from the major phenolics became evident with the concomitant appearance of two major peaks at 255 nm (Figure 6) in extracts from young leaves of young plants. Comparison with known spectra and standards suggested that the peaks represented 4-hydroxybenzoic acid glucoside (4-HBAG) and 4-hydroxybenzoic acid glucose ester (4-HBAGE). This identification was confirmed by positive and negative ion mass spectrometry (Figure 7A). In the late leaves from older flowering plants of line 201, these peaks were more prominent, and the average fresh weight content of 4-HBAG was >0.2% (Table 3). Mass spectrometry also demonstrated the presence of vanillic acid glucoside and vanillic acid glucose ester (Figure 7B). These peaks were noticeable in HPLC traces from late leaves of transgenic flowering plants, unopened flower buds, and brown seed capsules (Figure 6). Flower buds and seed capsules of line 201 transgenic plants accumulated high levels of 4-HBAG and 4-HBAGE, with the average level of 4-HBAG reaching ~0.5% of fresh weight in seed capsules (Table 3).

At 220 nm, four new peaks were identified in the leaves of transgenic plants. These were identified by mass spectrometry as vanillyl alcohol derivatives (Figure 7C) and 4-hydroxybenzyl alcohol glucosides (Figure 7D), and one (Figure 6, peak 10) had a retention time identical to that of a 4-hydroxybenzyl alcohol 4-*O*-glucoside (4-HBOHG) standard. The highest levels of 4-HBOHG were found in brown seed capsules and unopened flower buds, and the highest levels of all of the 4-hydroxybenzoic acid, 4-hydroxybenzyl alcohol, and vanillic acid derivatives were found in seed capsules of line 201 transgenic plants. The ratio of vanillic acid glucoside to 4-HBAG was higher in seed capsules (0.386) than in leaves (0.159) or green buds (0.07). Mass spectrometry confirmed that there was no detectable accumulation of protocatechuic aldehyde derivatives.

Another consequence of this production of new phenolic compounds in flowers was a decrease in petal anthocyanin levels. Line 201 transgenic plants, which exhibited the strongest reduction in chlorogenic acid, also presented the greatest reduction in anthocyanin. The mean level of anthocyanins in petals of line 201 syngenic plants was 75.4 ± 6.1 μmol/g fresh weight, whereas the line 201 transgenic plants contained 32.2 ± 0.9 μmol/g fresh weight. Together, these results suggest that expression of the HCHL gene caused a major diversion from the phenylpropanoid pathway to the production of novel 4-hydroxybenzaldehyde and vanillin derivatives.

DISCUSSION

The activity of the HCHL transgene in tobacco had two principal biochemical consequences: depletion of the major phenolic pools and accumulation of the novel chain-shortened C_6C_1 derivatives. Development of the abnormal morphology of the transgenic plants most likely was caused by the biochemical perturbation. There was a good correlation between the level of HCHL enzyme activity and the severity of the phenotype. The altered morphological phenotype of the HCHL transgenic plants was very similar to that of plants with reduced PAL activity. Cosuppression of PAL in transgenic tobacco by overexpression of a bean PAL construct led to stunted plants with curled leaves that developed localized lesions that were fluorescent in UV light and that were identical to the interveinal chlorosis seen with the HCHL plants (Elkind et al., 1990). In addition, flowers of one PAL-suppressed plant produced anthers with less pollen and decreased pollen viability (Elkind et al., 1990).

In the second generation of severely PAL-suppressed plants, the pattern of soluble phenolics in leaves was similar to that in control plants, but there was substantial depletion of chlorogenic acid and rutin (98 and 91%, respectively). Similarly, the severely affected HCHL line 201 showed up to 93 and 82% depletion of chlorogenic acid and rutin, respec-

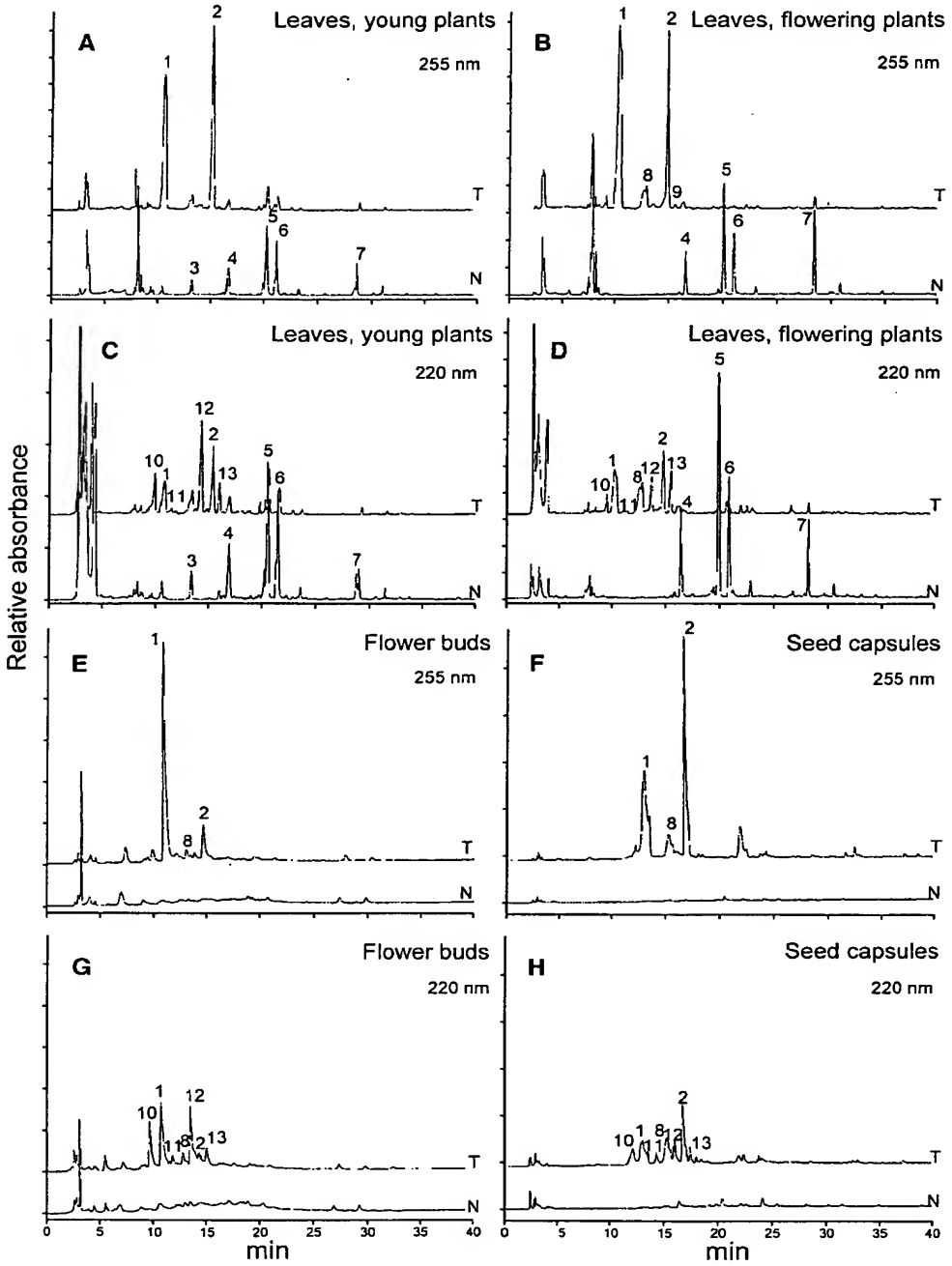

Figure 6. HPLC Analysis of Soluble Phenolics in Young Leaves, Unopened Flower Buds, and Seed Capsules of the Progeny of Line 201.

Phenolic profiles of transgenic (T) and syngenic (N) progeny of line 201 were compared at 255 and 220 nm in young leaves from young plants ([A] and [C]) and flowering plants ([B] and [D]), flower buds ([E] and [G]), and seed capsules ([F] and [H]). Key to peak identity: 1, 4-hydroxybenzoic acid glucoside; 2, 4-hydroxybenzoic acid glucose ester; 3, caffeoyl putrescine; 4, 3-caffeoylquinic acid; 5, chlorogenic acid; 6, 4-caffeoylquinic acid; 7, rutin; 8, vanillic acid glucoside; 9, vanillic acid glucose ester; 10, 4-hydroxybenzyl alcohol 4-*O*-glucoside; 11 and 13, vanillyl alcohol glucosides; 12, 4-hydroxybenzyl alcohol glucoside. Peaks were confirmed by mass spectrometry (see Figure 7).

Table 2. Accumulation of Major Soluble Phenolics in Young Leaves of HCHL Transgenic and Syngenic Progeny

		Mean ±SE (μg/g^{-1} fresh weight)[b]							
Plant Line[a]	*n*	3-CG	%	CGA	%	4-CG	%	Rutin	%
201, set 1, T	4	79 ± 12	40	200 ± 41	86	160 ± 30	76	9 ± 2	82
201, set 1, N	4	132 ± 16		1424 ± 45		641 ± 14		49 ± 11	
201, set 2, T	10	99 ± 8	63	238 ± 25	81	184 ± 16	70	25 ± 5	72
201, set 2, N	9	271 ± 27		1258 ± 180		604 ± 60		90 ± 8	
27, T	13	132 ± 8	14	500 ± 39	48	277 ± 16	33	32 ± 6	60
27, N	7	153 ± 4		970 ± 139		412 ± 41		20 ± 7	
39, T	12	114 ± 12	11	370 ± 50	76	209 ± 25	64	50 ± 10	49
39, N	1	128		1540		579		99	
13, T	5	83 ± 6	33	542 ± 35	48	240 ± 12	30	55 ± 15	26
13, N	5	124 ± 16		1033 ± 116		341 ± 23		74 ± 9	
201, set 1, TF	5	155 ± 12	68	124 ± 8	93	105 ± 4	86	63 ± 7	66
201, set 1, NF	3	493 ± 101		1715 ± 637		776 ± 147		186 ± 64	

[a] Line 201 was sown on two separate occasions (set 1 and set 2). T, transgenic; N, syngenic; F, taken during flowering.
[b] 3-CG, 3-caffeoylquinic acid; CGA, chlorogenic acid; 4-CG, 4-caffeoylquinic acid. Percentage change between syngenic and transgenic mean values.

tively. Because chlorogenic acid represents 60% and rutin represents 10% of the total phenolic pool in tobacco (Snook et al., 1986), expression of HCHL activity and PAL suppression both cause major depletion of total original phenolics in tobacco. Because an 88% depletion of chlorogenic acid caused by antisense inhibition of C4H did not result in interveinal chlorosis (Blount et al., 2000), this phenotypic abnormality seen with PAL suppression and expression of HCHL activity cannot be attributed solely to depletion of the main phenolic pool. Sense cosuppression of 4CL in tobacco (Kajita et al., 1996) and antisense suppression in Arabidopsis (Lee et al., 1997) did not lead to a leaf chlorosis phenotype. It is possible that some differences exist between the various types of transgenic plants, leading to subtly different spatial expression of the transgene, which may account for the varying morphological phenotypes with similar levels of phenolic pool depletion.

The reduction in pollen amount and viability seen in HCHL-expressing primary transformants and PAL-suppressed plants may be attributable to a reduction in the general flavonoid pathway and, more specifically, in flavonols. We did not quantitate flavonol content in the HCHL plants, but the major flavonoid rutin was depleted by up to 82% in leaves of line 201. The reduced fertility of the HCHL transgenic pollen in relation to the severe depletion of the flavonoid pool agrees with the proposed role of flavonoids in pollen development (Mo et al., 1992; van der Meer et al., 1992; Ylstra et al., 1992). In parallel with reduced pollen viability in HCHL-expressing plants, there was a severe reduction in anthocyanin content in petals of transgenic progeny (>70% in line 201). Some petals in PAL-suppressed tobacco were white, again suggesting extreme depletion of anthocyanins (Elkind et al., 1990). Similar floral phenotypes in tobacco were seen when the grapevine stilbene synthase was expressed from a CaMV 35S promoter (Fischer et al., 1997). Stilbene synthase uses malonyl-CoA and 4-coumaroyl-CoA as substrates to form the trihydroxystilbene resveratrol, and this rerouting of 4-coumaroyl-CoA into resveratrol resulted in flowers with inviable pollen and pale pink, almost white petals (Fischer et al., 1997).

Morphological and biochemical phenotypes similar to those caused by expression of the HCHL gene also were observed in other transgenic tobacco plants. A single tobacco transformant with reduced cinnamoyl-CoA reductase (CCR) activity exhibited a morphological phenotype that was very similar to the HCHL phenotype, with stunting, small leaves with curled margins, and interveinal discoloration or chlorosis (Piquemal et al., 1998). MYB transcription factors seem to repress the phenylpropanoid pathway after the cinnamic acid stage, and a series of transgenic tobacco plants were produced overexpressing the *Antirrhinum majus* Myb308 and Myb330 genes (Tamagnone et al., 1998). Myb308 plants were stunted, with reduced elongation of stem internodes during development and a striking leaf phenotype consisting of reduced leaf expansion and interveinal leaf necrosis. The leaf phenotype differed from that of HCHL plants in that the interveinal necrosis produced areas of white, dead tissue rather than the brown, senescent-like tissue seen with the HCHL plants. Flowers of Myb308 plants also had petals with reduced pigmentation, but the pollen was fertile. Leaf extracts of the Myb308 plants were deficient in all detectable soluble phenolics, with chlorogenic acid being reduced on average by 80% and flavonoid compounds being reduced to a lesser extent.

Lignin is a major component of secondary cell walls and is made by the polymerization of cinnamyl alcohols. Reduction of carbon flux through the phenylpropanoid pathway evident in the HCHL plants might be expected to have pro-

found effects on lignin formation. Coloration of the xylem ring was described previously in tobacco plants with reduced 4CL activity (Kajita et al., 1996), reduced CCR activity (Piquemal et al., 1998), and reduced cinnamyl alcohol dehydrogenase (CAD) activity (Halpin et al., 1994; Hibino et al., 1995; Yahiaoui et al., 1998). Xylem coloration also was observed in a loblolly pine mutant carrying a null CAD allele (MacKay et al., 1995), in poplar with downregulated CAD (Baucher et al., 1996), in a maize mutant of caffeic acid *O*-methyltransferase (COMT) (Vignols et al., 1995), and in transgenic trees with reduced COMT (van Doorsselaere et al., 1995; Tsai et al., 1998). A similar downregulation of COMT in tobacco did not lead to colored xylem (Atanassova et al., 1995), nor was it seen in the xylem ring of Myb308 and Myb330 plants (Tamagnone et al., 1998). The coloration of lignin may be attributable to the incorporation of nonlignin phenolics, with different phenolics imparting different colors (Higuchi et al., 1994; Piquemal et al., 1998; Yahiaoui et al., 1998).

The results we obtained with phloroglucinol staining indicate a quantitative and/or qualitative change in the lignin composition of HCHL plants. Similar decreases in phloroglucinol staining were observed in planta with modified phenylpropanoid and lignin metabolism, for example, in PAL- and CCR-suppressed tobacco plants (Bate et al., 1994; Piquemal et al., 1998). The most severely CCR-suppressed transformant exhibited collapsed vessels similar to those seen in HCHL plants (Piquemal et al., 1998), whereas Elkind et al. (1990) observed thinning of xylem cell walls in PAL-suppressed plants.

The singular aspect of using the Pseudomonas HCHL gene in tobacco was the extensive rerouting of carbon flow from phenylpropanoids into the synthesis of novel metabolites, consisting of the glucosides and glucose esters of the acid and alcohol derivatives of 4-hydroxybenzaldehyde and vanillin but with a complete absence of the protocatechuic forms. The extent of this rerouting was massive: the 4-HBAG in seed capsules of line 201 plants accumulated to approximately 4.5 mg/g fresh weight (i.e., 0.45% of the total fresh weight content). It is not surprising that the HCHL products are glucosylated, because complete glucosylation of the artificial metabolite 4-hydroxybenzoate produced in tobacco cell cultures by expression of the bacterial *ubiC* gene encoding chorismate pyruvate lyase was observed previously (Siebert et al., 1996). Two constitutive glucosyltransferase activities producing the glucoside and glucose ester forms of 4-hydroxybenzoic acid were able to glucosylate 4-hydroxybenzoate to produce glucosylated derivatives representing

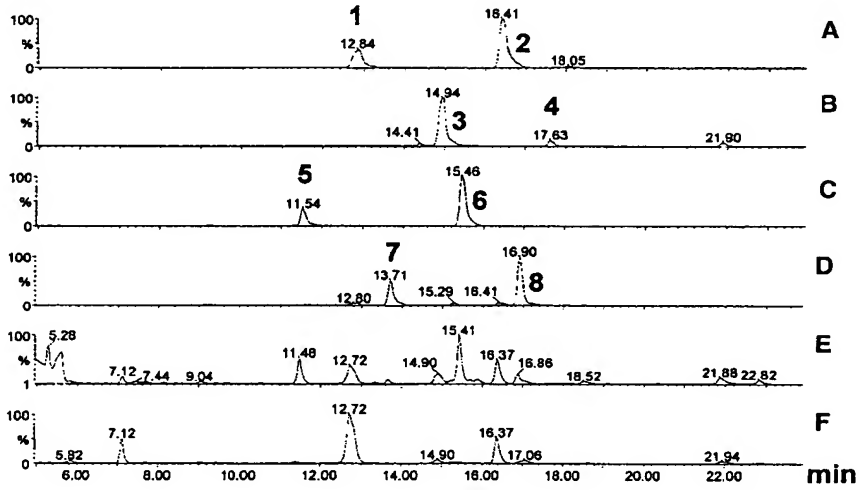

Figure 7. Liquid Chromatography–Mass Spectrometry Analysis of Benzaldehyde Derivatives.

Positive ion mass chromatograms of the sodium ([M + Na]$^+$) adducts of the molecules of mass 323 (peak 1, 4-hydroxybenzoic acid glucoside; peak 2, 4-hydroxybenzoic acid glucose ester) **(A)**; 353 (peak 3, vanillic acid glucoside; peak 4, vanillic acid glucose ester) **(B)**; 309 (peaks 5 and 6, 4-hydroxybenzyl alcohol glucosides) **(C)**; 339 (peaks 7 and 8, vanillyl alcohol glucosides) **(D)**; and the corresponding UV light chromatograms at 220 nm **(E)** and 254 to 256 nm **(F)**. The identities of these molecules were confirmed by their characteristic full scan spectra combined with the retention times of known standards. After subtraction of background peaks, the following characteristic mass spectra were obtained (only peaks with intensities >20% of base are reported): peak 1, 323 (100), 139 (67), 121 (22), 95 (70); peak 2, 323 (36), 139 (96), 121 (52), 95 (100); peak 3, 353 (100), 169 (69), 134 (30), 125 (72), 111 (35); peak 4, 353 (100), 125 (27); peak 5, 309 (14), 120 (22), 107 (44), 105 (100); peak 6, 309 (35), 107 (100); peak 7, 339 (70), 137 (100); peak 8, 339 (40), 137 (100).

Table 3. Quantitative Analysis of Soluble Phenolics in HCHL Transgenic Progeny[a]

Organ	Plant Line[b]	*n*	Mean ±SE (mg/g^{-1} fresh weight) 4-HBAG	4-HBAGE	4-HBOHG	VAG[c]
Leaves	201, set 1	5	0.54 ± 0.11	0.18 ± 0.03	0.06 ± 0.02	ND
	201, set 2	10	0.98 ± 0.05	0.36 ± 0.04	0.23 ± 0.01	ND
	27	12	0.56 ± 0.05	0.17 ± 0.01	0.08 ± 0.01	ND
	39	10	0.60 ± 0.05	0.25 ± 0.02	0.14 ± 0.01	ND
	13	5	0.32 ± 0.04	0.11 ± 0.02	0.07 ± 0.02	ND
	201, set 1, F	5	2.33 ± 0.20	0.59 ± 0.04	0.20 ± 0.00	0.37 ± 0.04
Buds	201, set 1	5	1.65 ± 0.10	0.15 ± 0.01	0.47 ± 0.02	0.12 ± 0.02
Capsules	201, set 1	3	4.58 ± 0.22	3.13 ± 0.36	1.03 ± 0.16	1.77 ± 0.20

[a] Mean accumulation of hydroxybenzaldehyde derivatives in young leaves, unopened flower buds, and mature seed capsules of transgenic HCHL progeny.
[b] Line 201 was sown on two separate occasions (set 1 and set 2). F, taken during flowering.
[c] VAG, vanillic acid glucoside; ND, not determined.

0.52% of the dry weight of the suspension cultures (Siebert et al., 1996) without a concomitant increase of the glucosyltransferase activities (Li et al., 1997). Less predictable in the HCHL plants was a complete absence of the aldehyde forms of the chain-shortened phenylpropanoids, with the acid form predominating.

The decreased accumulation of the vanillin-derived compounds compared with the 4-hydroxybenzaldehyde–derived compounds may be attributable to the kinetics of the metabolic diversion, because 4-coumaroyl-CoA is the precursor of caffeoyl-CoA and feruloyl-CoA. The vanillic acid derivatives accumulated to high levels in seed capsules, and the ratio between the vanillic acid forms and 4-hydroxybenzoate also was higher in seed capsules. Complete lack of the protocatechuic acid forms (i.e., the forms derived from caffeoyl-CoA) may result from metabolic channeling causing less access to the HCHL enzyme of the caffeoyl-CoA than of the feruloyl-CoA. Channeling was proposed previously for the phenylpropanoid and flavonoid pathways (Rasmussen and Dixon, 1999; Winkel-Shirley, 1999). Similar rapid conversion of 4-hydroxybenzaldehyde and vanillin in the HCHL plants to the acid and alcohol forms before glucosylation could explain the lack of glucosylated aldehydes. No benzyl alcohol glucoside derivatives were reported in tobacco cell lines or plants or in *Lithospermum erythrorhizon* hairy root cultures expressing the *Escherichia coli ubiC* gene (Siebert et al., 1996; Li et al., 1997; Sommer and Heide, 1998; Sommer et al., 1999). However, the HPLC analysis used absorbance at 254 nm, whereas the benzyl alcohol derivatives are detected optimally at 220 nm. Thus, we do not know whether 4-HBOG also was converted to the alcohol form in these systems.

Site-directed mutagenesis of the HCHL open reading frame to render the translation initiation context more typically eukaryotic (Kozak, 1986) did not produce a clear indication of whether the modification increased HCHL activity. Our sample size of the unmodified HCHL gene plants probably was too small to evaluate the effect of the modification confidently. Sommer and Heide (1998) performed a similar modification to the *E. coli ubiC* gene but did not determine the effect of this modification in planta.

The molecular response of the HCHL plants of line 201 was intriguing. Transcripts of PAL, C4H, and 4CL clearly were upregulated, CHS also was upregulated in general, but the PR2 gene was downregulated markedly. It is not possible to determine from our data whether the upregulation of the phenylpropanoid pathway and CHS was caused by feedback control by metabolite levels or was an indirect response to generalized cellular stress present in the HCHL plants. Stress induction of PAL, 4CL, and CHS transcripts has been reported in a number of systems (reviewed in Dixon and Paiva, 1995). However, the repression of the stress-inducible PR2 gene (Ward et al., 1991) suggests that the upregulation of the phenylpropanoid pathway and the CHS gene was attributable not to stress but to metabolic feedback. Transcript levels of PAL, C4H, 4CL, and CAD were repressed in Myb308 plants (Tamagnone et al., 1998), even though phenolic levels were depleted severely in these plants. The contrasting behavior of the HCHL and Myb308 phenylpropanoid pathway genes in the presence of a depleted phenolic pool may be explained by the proposed role of the Myb308 protein as a competitive inhibitor of other activating factors (Tamagnone et al., 1998), thereby suppressing transcription of the phenylpropanoid pathway genes.

In conclusion, expression of the Pseudomonas HCHL gene in tobacco caused massive rerouting of carbon flux from the phenylpropanoid pathway to novel C_6C_1 derivatives. The aldehyde products of HCHL activity were not detected, suggesting rapid conversion to the acid and alcohol forms. It is possible that glucosylation occurred after the dehydrogenation step, because no glucosylated aldehyde ester was detected. The ratio of vanillic acid to 4-hydroxybenzoic acid derivatives was higher in seed capsules, indicating

where future refinement of the metabolic engineering for flavor compounds should be targeted.

METHODS

Vector Construction

Molecular experiments were performed using standard protocols (Sambrook et al., 1989) or according to manufacturers' instructions. Restriction enzymes were obtained from Promega (Southampton, UK); general biochemicals were obtained from Sigma (Poole, Dorset, UK) unless stated otherwise.

Cosmid pFl1039 (Gasson et al., 1998) was restricted with EcoRI and BamHI to excise an 864-bp fragment encoding the 4-hydroxycinnamoyl-CoA hydratase/lyase (HCHL) enzyme, extending from −29 upstream of the ATG to 6 bp downstream of the stop codon. The fragment was ligated into the corresponding sites in pBluescript SK+ (Stratagene) and then excised with BamHI and HindIII and subcloned into the BamHI and HindIII sites of pJIT60, placing the sequence in a sense orientation between a cauliflower mosaic virus (CaMV) 35S RNA promoter with duplicated enhancer sequences and the CaMV termination sequence (Guerineau et al., 1992). The plasmid was restricted first with SacI and then partially with EcoRV, and the 2322-bp cassette was ligated into the SacI and SmaI sites of pBin19 (Bevan, 1984) to produce the construct pHCHL.

A second construct, pmHCHL, was produced based on a polymerase chain reaction (PCR)–amplified copy of the HCHL coding sequence incorporating a 1-bp change after the start codon (ATGAGC to ATGGGC) to improve the translational initiation context for plant expression (Kozak, 1986). Oligonucleotide primers (sense, 5′-ATCGCCATGGGCACATACGAAGGTC-3′; antisense, 5′-TCCTTCAGCGTTTATACGC-3′) to produce a 1-bp modification were synthesized in an ABI synthesizer (Applied Biosystems, Cheshire, UK). An 843-bp sequence (extending from −6 upstream of the ATG to 6 bp downstream of the stop codon) was amplified from pFl1039 with *Pfu* DNA polymerase (Stratagene) according to the manufacturer's instructions. The PCR product was ligated into the EcoRV site of pBluescript SK+, excised with EcoRI and SalI, and subcloned into the corresponding sites of pJIT60. The 2332-bp cassette was excised with EcoRV and KpnI and subcloned into the SmaI-KpnI sites of pBin19. pHCHL and pmHCHL constructs and an empty pBin19 vector were transferred into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating as described by Bevan (1984).

Plant Material and Transformation

Leaf discs of tobacco (*Nicotiana tabacum* cv Xanthi XHFD8) were transformed with *A. tumefaciens* (Horsch et al., 1985). Discs were incubated for 2 days on 1 × Murashige and Skoog (1962) medium (Melford Laboratories, Ipswich, UK) and then transferred to selective medium (1 × Murashige and Skoog medium incorporating 300 mg/L carbenicillin disodium [Melford Laboratories], 100 mg/L kanamycin sulfate, 10 mg/L dimethylaminopurine, and 1 mg/L indole acetic acid). After 3 to 5 weeks, plantlets were transferred to rooting medium (1 × Murashige and Skoog medium incorporating 300 mg/L carbenicillin and 50 mg/L kanamycin). Rooted plants were transferred to pots in a greenhouse (day temperature, 20°C; night temperature, 18°C; 16-hr day lit by high pressure sodium lamps). Plants were fed weekly with Solufeed high potash fertilizer (Kings Horticulture, Essex, UK).

Molecular Analysis of Plants

For RNA, DNA, and biochemical analyses, young leaves were taken from plants before flowering at nodes 9 and 10. Leaf samples also were taken during the early stages of flowering (approximately node 20). Total RNA and genomic DNA were extracted together using the method of Verwoerd et al. (1989). DNA and RNA gel blot analysis were performed as described (Sambrook et al., 1989) using Hybond-C extra nitrocellulose membrane (Amersham Pharmacia Biotech). RNA molecular weight markers were from Promega. The 843-bp PCR product excised from pBluescript SK+ was used as a probe to detect expression of the transgene. Expression of genes associated with phenylpropanoid and flavonoid biosynthesis was assessed using probes generously provided by Dr. Cathie Martin (John Innes Centre, Norwich, UK). A *Datura stramonium* ubiquitin sequence was generated by PCR from genomic DNA using primers derived from the tomato sequence (sense, 5′-ATGCAGATCTTCGTGAAAAC-3′; antisense, 5′-CTTAACCTTCTTCTTCTTCTGCTT-3′ [Hoffman et al., 1991]). Tobacco ubiquitin and β-1,3-glucanase (PR2) coding sequences were amplified by PCR from tobacco genomic DNA using oligonucleotide primers derived from published sequences (ubiquitin sense, 5′-TGGCTCAGGATGAACGCTGGC-3′; antisense, 5′-CATCTTTGAGACCTCAGTAGAC-3′ [Karrer et al., 1998]; PR2 sense, 5′-CATGCAAACAATTTACCATCAGACC-3′; antisense, 5′-CCAGGTTTCTTTGGAGTTCCTGCC-3′ [Ward et al., 1991]). DNA probes were gel purified (Qiaex II; Qiagen, Crawley, West Sussex, UK) and radiolabeled to high specific activity by random priming with High Prime (Roche Diagnostics, East Sussex, UK) using ^{32}P-dCTP (Amersham Pharmacia Biotech). Radiolabeled probe was separated from unincorporated nucleotides using a NICK column (Amersham Pharmacia Biotech). Filters were hybridized overnight in QuikHyb (Strategene) at 65°C, rinsed twice in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) at room temperature, and then washed twice for 30 min in 0.1 × SSC and 0.1% SDS at 65°C. Radioactivity was detected on x-ray film (RX; Fuji, Tokyo, Japan) using a Fuji BAS 1500 phosphorimager.

Assay of HCHL Activity and Protein Gel Blotting

The same ground frozen leaf powder used for RNA analysis was used to examine the HCHL enzyme activity and the soluble phenolic content. Enzyme activity in cell-free extracts was assessed by the method of Mitra (1999). Cell-free extracts were made at 4°C from 200 mg of frozen powder mixed with 20 mg of polyvinylpolypyrrolidone and 0.9 mL of extraction buffer (EB; 100 mM Tris-HCl, pH 8.5, 20 mM DTT, and 10 mM Na_2EDTA). Extracts were spun for 30 min at 13,000*g* and then applied to a PD10 column (Amersham Pharmacia Biotech) preequilibrated with 25 mL of EB. The column was washed with 1.6 mL of EB, and the sample was eluted in 1.26 mL of EB. Ten microliters of the eluate was incubated for 10 min at 30°C with 150 μM feruloyl-CoA in 100 mM Tris-HCl, pH 8.5, in a total volume of 100 μL. Total amounts of protein per reaction varied from 2 to 14 μg (usually 2 to 5 μg). The reaction was stopped with an equal volume of acidified methanol (12% glacial acetic acid and 88% methanol) and stored at −70°C.

Production of vanillin by HCHL activity in vitro was measured by gradient HPLC (apparatus from Spectra Physics, San Jose, CA) using a Lichrosorb 10-μm C18 column (25 cm × 4.6 mm; Phase Sep, Deeside, Clwyd, UK). Solvent A was 20 mM NaOAc, pH 6.0, solvent B was methanol, and the gradient ran for 50 min at 1.2 mL/min with the following concentrations: 0 min: 100% A, 0% B; 15 min: 90% A, 10% B; 40 min: 50% A, 50% B; 45 min: 30% A, 70% B; and 50 min: 100% A, 0% B. The column eluant was monitored using a Spectra Focus scanning detector (Spectra Physics). A vanillin standard was used to calculate the response factor in this system.

Protein concentration of the PD10 samples was determined by the method of Bradford (1976) with the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) using BSA as a standard. Expression of the HCHL protein in transgenic plants was confirmed by protein gel blotting using a polyclonal antibody (Mitra, 1999). Total protein was extracted from frozen, powdered leaf material by shaking overnight at 4°C in 0.1 M Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, and 0.001% bromphenol blue at 40 mg (fresh weight)/mL. DTT was added to 0.5%, and samples were heated for 5 min at 95°C and spun to pellet debris before electrophoresis through a 12% polyacrylamide gel using reagents supplied by National Diagnostics (from Flowgen, Lichfield, Staffordshire, UK). Protein gel blotting was performed as described by Carter et al. (1997) using ProBlot membrane (Applied Biosystems) and the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium liquid substrate system (Sigma). A duplicate gel was stained to visualize the proteins and the size marker (Gibco BRL, Paisley, UK) using silver stain reagents (Sigma).

HPLC and Liquid Chromatography–Mass Spectrometry Analysis of Soluble Phenolics

Soluble phenolics were extracted by vortexing 30 mg of frozen, powdered leaf material briefly in 300 μL of 70% methanol. After the addition of 100 μL of distilled water, the extract was vortexed again and then spun for 10 sec at 13,000*g* to pellet the debris. The extract was analyzed by HPLC using a Columbus 5-μm C18 column (25 cm × 4.6 mm; Phenomenex, Macclesfield, Cheshire, UK). Solvent A was 1 mM trifluoroacetic acid, solvent B was acetonitrile, and the gradient was run for 65 min at a flow rate of 1 mL/min with the following concentrations: 0 min: 100% A, 0% B; 50 min: 60% A, 40% B; 60 min: 30% A, 70% B; and 65 min: 100% A, 0% B.

Combined liquid chromatography–mass spectrometry was performed using a Hewlett-Packard 1050 HPLC system (Agilent Technologies, Stockport, UK) and a Quattro II mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray electrospray ion source. The HPLC conditions were as described for the analysis of soluble phenolic compounds. The flow exiting the HPLC column (1 mL/min) was split 8:1 between the diode array detector and the mass spectrometer ion source. In positive ion mode, the electrospray capillary voltage was set to 3.5 kV and the cone voltage was set to 29 V; the corresponding voltages in negative ion mode were 2.87 kV and 29 V, respectively. The source temperature was set to 140°C, and the desolvation temperature was set to 350°C. Mass spectra were scanned, at unit mass resolution, from mass-to-charge ratio of 50 to 1100 at a rate of 2 sec per scan, with an interscan time of 0.1 sec. Diode array UV light spectra were recorded between 190 and 400 nm, with a step interval of 2 nm and scan interval of 0.1 min. All data were processed and displayed using MassLynx software, version 3.4 (Micromass).

Standards for 4-hydroxybenzoic acid glucoside (4-HBAG) and 4-hydroxybenzoic acid glucose ester (4-HBAGE) were identified by comparison with standards synthesized by Dr. Shigeo Tawaka (Kyoto University, Japan) and kindly provided by Prof. Dr. Lutz Heide (University of Tübingen, Tübingen, Germany). The response factors of these standards in this system were determined by alkaline hydrolysis (4-HBAGE) or β-glucosidase digestion (4-HBAG and vanillic acid glucoside) of known volumes of standards to liberate free 4-hydroxybenzoic acid or vanillic acid, which were then quantified. Hydrolysis by the method of Cooper-Driver et al. (1972) was used to remove glucose from the 4-hydroxybenzyl alcohol glucosides, and the liberated alcohol was compared with a 4-hydroxybenzyl alcohol standard. A vanillic acid glucoside standard was kindly provided by the Takasago International Corporation (Tokyo, Japan).

Gas Chromatography–Mass Spectrometry

Gas chromatography–mass spectrometry was performed on a Fisons Instruments (Altrincham, UK) Trio 1-S mass spectrometer equipped with a Hewlett-Packard 5890B Series II gas chromatograph. Injections of 1 μL of sample were made in splitless mode with an injector temperature of 250°C and with the purge valve on after 1 min. The column used was from J&W Scientific (Folsom, CA; DB5-MS, 15 m, 0.32-mm i.d., 0.25-μm film thickness). The program was 60°C for 1 min, 10°C/min until 250°C was reached, and then hold for 10 min. Samples were ionized by electron ionization at 70 eV and analyzed by cyclic continuous scanning from mass-to-charge ratio of 35 to 600 at a rate of 0.9 sec per scan, with an interscan time of 0.1 sec.

Measurement of Anthocyanin

Anthocyanin was extracted by the method of Martin et al. (1985). Petals from tobacco flowers were weighed, cut into small pieces, and then extracted overnight at 4°C in sealed bottles containing 10 mL of 97% methanol and 3% HCl. The peak absorbance at 530 nm was measured in a spectrophotometer at 530 nm, and the anthocyanin content was calculated given that 1 OD = 33 mM anthocyanin (Martin et al., 1985).

Microscopic Examination of Tobacco Stems

Hand-cut, unstained sections of tobacco stems above leaf node 12 were taken from second generation transgenic and syngenic plants of line 201 during mid flowering. The vascular ring was observed under a dark field (Wild Heerbrugg stereomicroscope [Wild Leitz, Heerbrugg, Switzerland]). Cellular structure was examined by light microscopy (Leitz Ortholux II microscope [Leitz, Wetzlar, Germany] with a HBO 50-W mercury arc lamp and an exciter and barrier filter combination with transmission of 450 to 490 nm and >515 nm, respectively). Phloroglucinol staining was performed using the method of Speer (1987), and sections were viewed by light microscopy.

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Genetic Engineering of Plant Secondary Metabolism[1]

Accumulation of 4-Hydroxybenzoate Glucosides as a Result of the Expression of the Bacterial *ubiC* Gene in Tobacco

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The *ubiC* gene of *Escherichia coli* encodes chorismate pyruvate-lyase, an enzyme that converts chorismate into 4-hydroxybenzoate (4HB) and is not normally present in plants. The *ubiC* gene was expressed in *Nicotiana tabacum* L. plants under control of a constitutive plant promoter. The gene product was targeted into the plastid by fusing it to the sequence for the chloroplast transit peptide of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Transgenic plants showed high chorismate pyruvate-lyase activity and accumulated 4HB as β-glucosides, with the glucose attached to either the hydroxy or the carboxyl function of 4HB. The total content of 4HB glucosides was approximately 0.52% of dry weight, which exceeded the content of untransformed plants by at least a factor of 1000. Feeding experiments with [1,7-$^{13}C_2$]shikimic acid unequivocally proved that the 4HB that was formed in the transgenic plants was not derived from the conventional phenylpropanoid pathway but from the newly introduced chorismate pyruvate-lyase reaction.

Genetic engineering has allowed the production of plants with an altered content of secondary metabolites. Because secondary metabolites are important in the defense of plants against pathogens, such engineered plants may show an increase in resistance against pathogens. For example, the expression of a stilbene synthase from *Vitis vinifera* in tobacco (*Nicotiana tabacum*) led to the accumulation of stilbenes and thereby to an increased resistance to *Botrytis cinerea*, providing direct evidence of the role of stilbenes as phytoalexins (Hain et al., 1993). In other experiments the accumulation of secondary metabolites, which are already produced in the untransformed plants, has been increased by the overexpression of structural genes encoding biosynthetic enzymes. Expression of hyoscyamine 6-hydroxylase in *Atropa belladonna* plants, for example, led to a strong increase of scopolamine production in the transgenic plants (Yun et al., 1992), and expression of a bacterial Lys decarboxylase in tobacco increased the production of the diamine cadaverine (Fecker et al., 1993). Such experiments may prove useful for the production of secondary metabolites of pharmaceutical importance, both by intact plants and by plant cell cultures.

The commercial production of a pharmaceutical substance by plant cell culture has already been realized on an industrial scale in the case of shikonin, a naphthoquinone pigment with antibacterial, antiphlogistic, and wound-healing properties that is obtained from cell cultures of *Lithospermum erythrorhizon* (Tabata and Fujita, 1985). Shikonin is biosynthetically derived from 4HB and geranylpyrophosphate (Heide and Tabata, 1987). Using feeding experiments with [1,7-$^{13}C_2$]shikimic acid (Fig. 1; Heide et al., 1989), we have shown that the production of 4HB in these cell cultures proceeds exclusively via phenylpropanoid intermediates, and it has been proposed that most benzoic acids as well as ubiquinones are derived from the phenylpropanoid pathway in plants (Pennock and Threlfall, 1983). Furthermore, the conversion of the phenylpropanoid precursors to 4HB was recently characterized (Löscher and Heide, 1994), showing that the reaction sequence from chorismate to 4HB in plants involves up to 10 successive enzymatic reaction steps in cell cultures of *Lithospermum erythrorhizon*.

Escherichia coli, on the other hand, possesses a simpler biosynthetic route to 4HB, which involves the direct conversion of chorismate to 4HB by CPL (Fig. 1b). The cloning of *ubiC*, the gene encoding CPL, was recently reported by our group (Siebert et al., 1992, 1994) and by Nichols and Green (1992). The protein was overexpressed, purified, characterized, and shown to be a soluble protein of 19 kD. It has a K_m value for chorismate of 6.1 μM, a pH optimum at 7.5, and does not require cofactors.

In this study we have expressed the *ubiC* gene in tobacco, thereby introducing a single-step process for the production of 4HB in plants. Chorismate, the substrate of the *ubiC* gene product, is an intermediate of the shikimate pathway. In plants this pathway is localized in the plastid, and the existence of an additional shikimate pathway in the cytosol is controversial (Hrazdina and Jensen, 1992). Therefore, the *ubiC* gene product was targeted to plant plastids by fusing it to a sequence for the chloroplast transit peptide of the small subunit of Rubisco. Expression of these constructs in

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Abbreviations: CPL, chorismate pyruvate-lyase; 4HB, 4-hydroxybenzoic acid; MS, Murashige-Skoog.

tobacco led to the accumulation of 4HB glucosides in the transgenic plants.

MATERIALS AND METHODS

Chemicals and Enzymes

Chorismate was purchased as the barium salt from Sigma, and [1,7-$^{13}C_2$]shikimic acid was synthesized as described by Cho et al. (1992). *Taq* polymerase was obtained from Pharmacia or Amersham, and the T4-DNA-ligase and Klenow enzymes were purchased from Boehringer Mannheim. The Sequenase Kit, version 2.0, was obtained from United States Biochemical, and [^{35}S]dATPαS was purchased from Amersham. PCR primers were synthesized by Michael Weichsil Gartner Biotech (Ebergsberg, Germany).

Bacteria, Plants, Cell Cultures, and Media

Escherichia coli XL1Blue, AN92, and JM109 were described by Bullock et al. (1987), Young et al. (1971), and Yanish-Perron et al. (1985), respectively, and *Agrobacterium tumefaciens* strain LBA4404 was described by Hoekema et al. (1983). Cell cultures of tobacco (*Nicotiana tabacum*) were established by cutting sterile-grown seedlings into 1-cm pieces and culturing them on MS medium (Murashige and Skoog, 1962) with callus-inducing hormones (containing per L: 4.46 g of MS salts [Sigma], 30 g of Suc, 0.05 mg of kinetin, 0.5 mg of 2,4-D, and 10 g of Bacto Agar [Difco, Detroit, MI], pH 5.7). After 4 to 6 weeks the generated callus tissues were transferred into 300-mL conical flasks; each flask contained 75 mL of liquid MS medium (containing per L: 4.46 g of MS salts, 30 g of Suc, 1 mg of NAA, and 0.2 mg of 6-benzylaminopurine, pH 5.7). The cultures were maintained in the dark at 25°C and 80 rpm, with subculturing at 14-d intervals. Germination medium contained per L: 4.6 g of MS salts with minimal organic contents [Sigma], 30 g of Suc, and 8 g of Bacto Agar, pH 5.7.

Plasmids and DNA Manipulation

pROK1 and pUC18 were described by Baulcombe et al. (1986) and Yanish-Perron et al. (1985), respectively; pUbiC and pALMU1 were described by Siebert et al. (1994) and Siebert et al. (1992), respectively; and pTSS1-91(#2)-IBI was described by Sugita et al. (1987). DNA manipulation was performed according to the method of Sambrook et al. (1989). The sequencing reactions were performed using the kit from United States Biochemical according to the manufacturer's instructions.

Construction of Plasmids and Transformation of Plants

A PCR product was obtained from plasmid pALMU1 (Siebert et al., 1992) with the primers ubiC-3F (5'-TTA TGT AAC GGA GAG TTC GGC ATG-3') and ubiC-R (5'-ATT CTG CGT CAG ACT CCA CTC CAT ATT TTT TTC CTC TTA-3') and was ligated into the *Sma*I site of pUC18 to give plasmid pUbiC. The correct orientation of the insert was confirmed by restriction analysis. The PCR product obtained from the pTSS1-91(#2)-IBI (Sugita et al., 1987) with the primers RBCS-F (5'-CGGAATTCAGATCTAGACAAT ATG GCT TCC TCT G-3') and RBCS-0R (5'-TGGGGTAC-CGC GCA GCT AAC TCT TCC AC-3') was digested by *Eco*RI and *Kpn*I. This insert was ligated into plasmid pUbiC, digested with *Eco*RI and *Kpn*I, resulting in plasmid pTP-UbiC. Subsequently, the plasmid pTP-UbiC was digested by *Bgl*II and *Sal*I. The resulting fragment, containing the TP-*ubiC* fusion gene, was ligated to the *Bam*HI site of pROK1 with the compatible *Bgl*II site. The noncompatible *Sal*I and *Bam*HI ends were filled in with Klenow polymerase and blunt end-ligated, resulting in the construction of plasmid p35S-TP-UbiC. The correct orientation of the insert was confirmed by restriction analysis. Transformation of *A. tumefaciens* strain LBA4404 was carried out as described by Höfgen and Willmitzer (1988), and leaf disc transformation and regeneration of *N. tabacum* was performed according to the method of Baumann et al. (1987), which was modified according to the method described by Horsch et al. (1985).

Genomic DNA Gel Blots, RNA Gel Blots, and Immunoblots

DNA was extracted from tobacco suspension cultures as described by Dellaporta et al. (1983). To detect the presence of the *ubiC* gene, PCR analysis was done according to standard procedures (Sambrook et al., 1989) using the

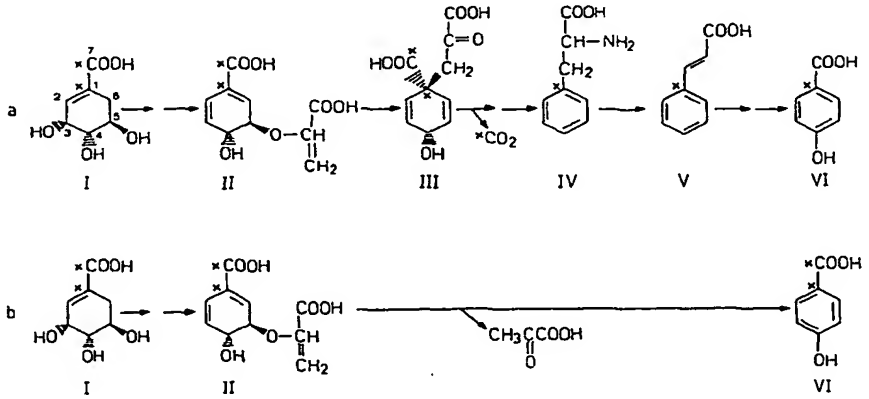


Figure 1. Pathway for biosynthesis of 4-hydroxybenzoate in plants (a) and *E. coli* (b). I, Shikimic acid; II, chorismic acid; III, prephenic acid; IV, phenylalanine; V, cinnamic acid; VI, 4HB. The positions used for ^{13}C labeling (see text) are marked with an x.

primers: 5′-CAC ACC CCG CGT TAA CGC-3′ and 5′-CAT TCT GCG TCA GAC TCC-3′. The calculated size of the amplified band was 531 bp.

Genomic DNA gel blots were prepared by the digestion of tobacco genomic DNA with *Hin*dIII and *Eco*RI, the separation of DNA fragments on an agarose gel, and the transfer to Hybond-N^+ membranes (Amersham). A PCR product was obtained from pUbiC with the primers mentioned above, labeled with [α-^{32}P]dCTP, and used as a probe. Prehybridization and hybridization were performed at 65°C in Rapid-hyb-buffer (Amersham).

RNA was extracted from tobacco suspension cultures as described by Logemann et al. (1987). RNA was separated on a 1.5% agarose gel with 6% formaldehyde following standard procedures (Sambrook et al., 1989). Transfer to Hybond-N^+ membranes and detection were carried out as described below.

Bacterial and plant enzyme extracts were prepared for immunoblots as described above. Plant extracts were applied to a HiTrap blue column (Pharmacia; 10-mL bed volume), and CPL was eluted with a gradient of 0.2 to 2 M NaCl in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM EDTA. Fractions containing the CPL activity, or the corresponding fractions obtained from untransformed control plants, were subjected to SDS-PAGE and blotted onto nitrocellulose membranes according to standard procedures (Sambrook et al., 1989). The UbiC protein was detected using specific antibodies (Siebert et al., 1992) and the BM chemoluminescence western blotting kit (Boehringer Mannheim).

Preparation of Enzyme Extracts and Determination of CPL Activity

Bacterial enzyme extracts were prepared as described by Siebert et al. (1992, 1994). For the extraction of enzymes from plants, tobacco leaves (2 g) were homogenized in an ice-cooled mortar with 2.6 mL of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% β-mercaptoethanol, and 200 mg of polyvinylpolypyrrolidone. After the sample was centrifuged (20,000*g*, 10 min) the supernatant was passed over a Sephadex G-25 (Pharmacia) column that was equilibrated with 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 200 mM NaCl; the pH of this buffer was adjusted to pH 8.0 at 37°C. Protein content was determined as described by Bradford (1976), and CPL activity in bacterial extracts was assayed according to the method of Siebert et al. (1992). A modified assay was used for plant extracts, containing in a total volume of 500 μL: 100 nmol of purified chorismate (Siebert et al., 1994), 25 μmol of Tris-HCl, pH 8.0, 5 μmol of EDTA, 100 μmol of NaCl, and 50 μg of protein. Incubations were carried out at 37°C for 15 min, after which reactions were stopped and analyzed by HPLC as described by Siebert et al. (1992). A correction was made for the amount of 4HB formed by the chemical decomposition of chorismate.

Fractionation of Enzyme Extracts from Tobacco Cell Cultures

Cytosolic and plastid fractions were prepared and assayed for the respective marker enzymes, alcohol dehydrogenase, and shikimate dehydrogenase, as described by Sommer et al. (1995).

Isolation and Structural Elucidation of 4HB Glucosides

Fifty grams of fresh leaves was extracted with 120 mL of methanol, and the solution was subsequently concentrated to 16 mL, acidified to pH 2.0 (1 N HCl), and extracted twice with an equal volume of diethyl ether. The ether phase was used for the determination of free 4HB (see below). Aliquots of the aqueous phase were subjected to HPLC on a Multospher 120 RP-18 column (C & S Chromatographie Service, Düren, Germany) using a gradient from 15 to 50% methanol in water:formic acid (99:1, v/v). The two dominant compounds detected at 254 nm were collected and repurified on the same HPLC column using a gradient from 0 to 50% methanol in water:formic acid (99:1, v/v). Evaporation of the solvent produced compounds **1** (10 mg) and **2** (3 mg), which were analyzed by UV spectroscopy and NMR.

The properties of compound **1** [4-*O*-(1-β-D-glucosyl)benzoic acid] were as follows. By UV spectroscopy: λmax: 255 nm (H_2O, pH 6.0); by ^{1}H NMR: (250 MHz) δ_{ppm} (D_2O), 3.1 to 3.8 (m, 6H), 5.09 (d, 1H, J = 7.3 Hz, H-1′), 7.02 (d, 2H, J = 9 Hz, H-3,5), 7.82 (d, 2H, J = 9 Hz, H-2,6); by ^{13}C NMR (100 MHz): δ_{ppm} (CD_3OD): 62.5 (C-6′), 71.3 (C-4′), 74.8 (C-2′), 77.9 (C-3′), 78.3 (C-5′), 101.7 (C-1′), 117.1 (C-3, 5), 125.7 (C-1), 132.7 (C-2, 6), 162.8 (C-4), 169.6 (COO—).

The properties of compound **2** (4HB 1-β-D-glucosyl ester) were as follows. By UV spectroscopy: λmax: 264 nm (H_2O, pH 6.0); by ^{1}H NMR (400 MHz) δ_{ppm} (DMSO-d_6), 3.1 to 3.8 (m, 6H), 5.54 (d, 1H, H-1′), 6.88 (d, 2H, J = 9 Hz, H-3,5), 7.87 (d, 2H, J = 9 Hz, H-2,6); by ^{13}C NMR (100 MHz): δ_{ppm} (DMSO-d_6), 60.6 (C-6′), 69.6 (C-4′), 72.6 (C-2′), 76.5 (C-3′), 77.9 (C-5′), 94.6 (C-1′), 115.4 (C-3,5), 119.8 (C-1), 131.9 (C-2,6), 162.4 (C-4), 164.4 (COO—).

Determination of 4HB Content

To determine the total 4HB content, 1.5 g of fresh leaves was homogenized with liquid nitrogen and extracted at room temperature with methanol (5 mL) using an Ultra-Turrax (Janke and Kunkel, Staufen, Germany) at 8000 rpm for 1.5 h, and the pellet was re-extracted with 2 mL of methanol. The combined extracts were centrifuged at 13,000*g* for 15 min; the supernatant was evaporated and the residue was hydrolyzed with 0.1 N HCl at 80°C for 2 h. 4HB was extracted with ethyl acetate (2 × 1 mL), and the organic phase was evaporated and analyzed for 4HB by HPLC using the same method as for the CPL assays.

To determine the amount of free 4HB, the ether phase obtained during the isolation of the 4HB glucosides (see above) was examined by HPLC, using the same conditions as for the CPL assay. For analysis of the 4HB content in the cell walls, the methanol-insoluble residue was subjected to acid (1 N HCl) or alkaline (1 N NaOH) hydrolysis for 2 h at 95°C and extracted with ethyl acetate, and the organic layer was analyzed by HPLC.

Segregation of the Antibiotic-Resistant Phenotype in the First Self-Fertilized Filial Generation

A total of 468 seeds that were obtained from transformant II was sterilized by 5% $Ca(OCl)_2$, supplemented with 1% Tween 20 (Sigma) for 15 min, and placed on germination medium containing 100 mg/L kanamycin; 95.7% of the seeds germinated. A ratio of 1:3.2 (sensitive:resistant) was observed, and similar results were obtained from the other transformants.

Feeding of [1,7-$^{13}C_2$]Shikimic Acid

A total of 14.08 mg [1,7-$^{13}C_2$]shikimic acid was added to eight flasks, each containing 75 mL of MS medium with transgenic tobacco suspension cultures of transformant II, 4 d after inoculation. After 7 d of incubation (25°C in the dark at 80 rpm) the cells (150 g) were harvested and homogenized in 350 mL of ice-cooled methanol. The methanolic solution was filtered and evaporated to 70 mL, and after the addition of 80 mL of water the solution was extracted with 3 × 100 mL of diethyl ether, and the organic phase was discarded. The aqueous solution was evaporated to dryness, and the residue was taken up in 40 mL of methanol, sonicated, separated from insoluble parts by filtration, and evaporated to dryness. This procedure was repeated; the filtrate was evaporated to dryness after the addition of 4 g of silica gel and applied to a silica gel column (30 g) in ethyl acetate. Elution was carried out with a gradient of methanol (0–20%) in ethyl acetate. Fractions of 20 mL were collected and analyzed for 4-*O*-(1-β-D-glucosyl)benzoic acid by TLC (silica gel F_{254}, acetone:chloroform:formic acid, 5:4:1, v/v), using UV detection. Fractions containing 4-*O*-(1-β-D-glucosyl)benzoic acid were pooled and evaporated to dryness, and the residue was purified by HPLC (RP-18 column; water:methanol:formic acid, 183:15:2, v/v). The fractions containing 4-*O*-(1-β-D-glucosyl)benzoic acid were pooled and evaporated to dryness, and the crystalline residue was recrystallized from 0.2 mL of water to yield 7.6 mg of white crystals.

RESULTS AND DISCUSSION

Construction of Vectors for *ubiC* Expression in Plants

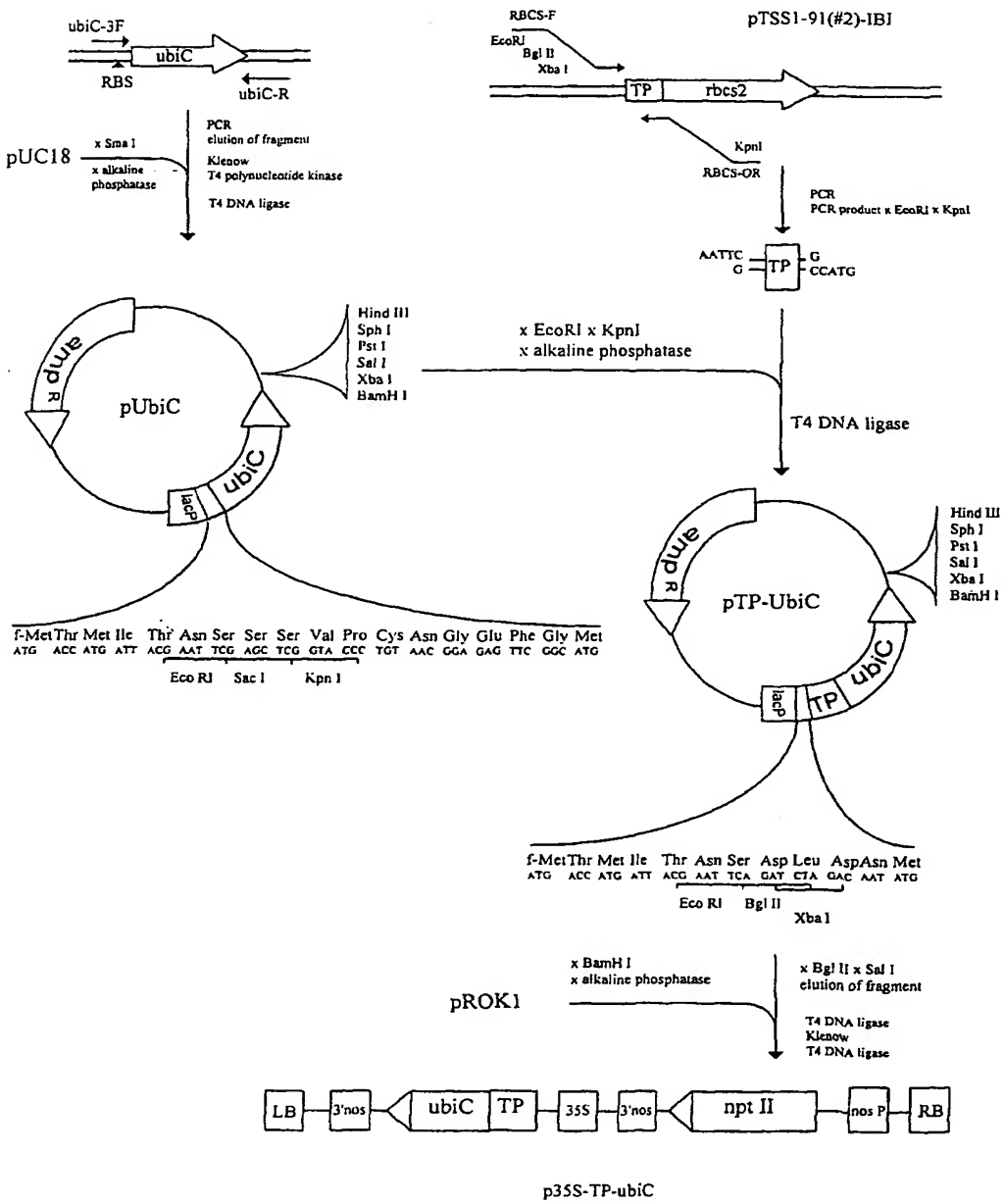
To achieve expression of the bacterial *ubiC* gene in plants, the gene was fused to a plant promoter, to a plant terminator, and to a transit peptide for plastid targeting. Because no suitable restriction site was available upstream of the *ubiC* start codon in the original sequence of *E. coli*, the structural gene was amplified by PCR, and the PCR product was ligated into a pUC18 vector. From this construct, termed pUbiC, the CPL was expressed as a fusion protein with the first 17 amino acids of the *lacZ* gene product (Fig. 2). Subsequently, the gene for the transit peptide of the Rubisco small subunit, rbcs-2A (Pichersky et al., 1986), was amplified by PCR as described in "Materials and Methods." The PCR product was ligated into the pUbiC vector (Fig. 2), and the resulting construct was termed pTP-UbiC. Sequencing of the entire insert confirmed that no mutations had occurred during the PCR amplifications.

To confirm the enzymatic activity of the proteins expressed from these gene fusions, the recombinant plasmids were transformed into the *E. coli* mutant AN92 (Young et al., 1971), which lacks chorismate mutase and therefore allows the accurate measurement of CPL activity (Lawrence et al., 1974). Both constructs caused an increase of CPL activity by 2 or 3 orders of magnitude, as compared with the empty vector, indicating that the expressed proteins were enzymatically active (Table I). However, the construct including the transit peptide gave lower activities than the construct with *ubiC* alone, indicating that fusion with the transit peptide reduces enzymatic activity. However, this finding should not present an obstacle to an expression with high activity in plants, because the transit peptide is expected to be removed after import into the plastid. Using an antibody obtained against UbiC (Siebert et al., 1992) we carried out immunoblots for the fusion proteins produced from pUbiC and pTP-UbiC (Fig. 3A, lanes 3 and 4). The apparent M_r observed for the proteins exceeded the calculated M_r by approximately 10%, which is still in reasonable agreement with the expected value. The lower band observed in lane 4 may be attributed to a degradation product of the original LacZ-TP-UbiC fusion.

ubiC Expression in Transgenic Plants

To achieve transformation into tobacco the gene construct was transferred into the binary vector pROK1 (Baulcombe et al., 1986), which contains a 35S promoter and a *nos* terminator for foreign gene expression, and a kanamycin-resistance gene as a selectable marker (Fig. 2). The binary vector construct was used for *A. tumefaciens* transformation of leaf discs of *N. tabacum* cv Petite Havanna (SR1). Four transgenic plants (I-IV) were regenerated under a selection pressure for antibiotic resistance, with no phenotypic changes observed in comparison with the untransformed control. These plants were examined by PCR (data not shown) and by genomic DNA gel blot analysis (Fig. 3B), and the bacterial *ubiC* gene, which was absent in the untransformed control, was present. Transgenic plant seeds obtained by self-pollination were germinated under sterile conditions in the presence of kanamycin and were examined for segregation of the antibiotic-resistance phenotype. A segregation of approximately 1 (sensitive) to 3 (resistant) was observed (see "Materials and Methods"), as expected for a monogenic dominant trait. Therefore, we concluded that the antibiotic-resistance gene had been stably integrated into the genome at one locus.

RNA gel blots showed, upon hybridization with a *ubiC* probe, a single band of the expected size (approximately 1100 bases), whereas no signal was observed with SR1 control plants (Fig. 3C). The transgenic plants were investigated for CPL activity, and the results are shown in Table I. The transformants had high enzyme activity, exceeding those of a wild-type *E. coli* by a factor of up to 80. As expected, different transformants showed different activi-



p35S-TP-ubiC

Figure 2. Construction of vectors for the expression of the bacterial *ubiC* gene in tobacco. The nucleotide sequences of the first 18 codons of the *lacZ-ubiC* gene fusion and of the first 12 codons of the *lacZ*-transit peptide-*ubiC* fusion are given. The last codons represent the native start codon of *ubiC* or of the transit peptide, respectively. RBS, Ribosomal binding site; lacP, *lacZ promoter; amp*^{R}, *ampicillin-resistance gene; TP, transit peptide sequence of the small subunit of Rubisco;* nptII, neomycin phosphotransferase gene; 35S, cauliflower mosaic virus 35S promoter; nos P, *nos* promoter; 3'nos, *nos* terminator; LB, left border; RB, right border; f-Met, *N*-formyl-methionine. RBSC-F and RBSC-OR, forward and reverse primer (see "Materials and Methods").

ties, presumably because of integration into different positions of the genome.

Immunoblot analysis (Fig. 3A) of the transgenic plants showed a single band at the appropriate position for UbiC, rather than for the TP-UbiC fusion, suggesting that the TP-UbiC fusion protein had been processed by the stromal protease of the plastid. In addition, cell-fractionation experiments with cell-suspension cultures obtained from the transgenic tobacco indicated that CPL activity correlated with the plastid marker enzyme shikimate dehydrogenase

Table I. *CPL activity in E. coli AN92 and in N. tabacum after expression of ubiC gene constructs*

The value obtained with pUC18 is expected to correspond to the activity in a wild-type *E. coli*. Enzyme activity data are mean values ± SD of four independent determinations. 4HB content data are mean values ± SD of at least seven independent determinations.

Transformant	CPL Activity	Content of 4HB Derivatives in Leaves
	pkat/mg protein	*μmol/g dry wt*
E. coli::pUC18[a]	2.7 ± 0.8	–
E. coli::pUbiC(+)[b]	3923 ± 263	–
E. coli::pUbiC(−)[c]	3.8 ± 1.4	–
E. coli::pTP-UbiC	675 ± 66	–
N. tabacum SR1	<1.0	<0.02
N. tabacum transgenic plants		
I	207.8 ± 21.3	17.2 ± 2.0
II	148.4 ± 12.7	15.0 ± 4.7
III	138.6 ± 10.1	14.6 ± 4.6
IV	38.4 ± 8.8	5.9 ± 3.8

[a] Empty vector. [b] Vector with *ubiC* in correct orientation. [c] Vector with ubiC in opposite orientation.

rather than with the cytosolic marker enzyme alcohol dehydrogenase (Table II).

Accumulation of 4-Hydroxybenzoate Derivatives in the Transgenic Plants

Because chorismate, as a metabolite of the shikimic acid pathway, is present in the plastids, the expression of active CPL in this compartment should give rise to the formation of 4HB. Therefore, leaves of the transgenic plants were examined for their 4HB content. Transgenic plants accumulated up to 17.2 μmol 4HB/g dry weight, compared with <0.02 μmol/g dry weight in the untransformed control, and the total content of 4HB detected after acid hydrolysis in the different transgenic plants correlated with the CPL activity of these transformants (Table I).

Without acid hydrolysis very little 4HB (0.2 μmol/g dry weight) was detected in the transgenic plants, showing that the plants accumulate 4HB mainly in the form of derivatives. To identify these 4HB derivatives fresh leaves were extracted with methanol. Two compounds (1 and 2) were detected, which were present in the transgenic plants but not in the untransformed control; these compounds were isolated by HPLC. Upon acid hydrolysis (1 N HCl for 60 min at 95°C) both compounds produced 4HB, which was identified by TLC, HPLC, and UV spectroscopy. The presence of the 4HB moiety was also confirmed by ^{1}H NMR and ^{13}C NMR analysis of compounds 1 and 2, which revealed the signals of a benzoic acid substituted at C-4 (see "Materials and Methods"). In addition, both compounds showed the signals of Glc. The ^{13}C NMR spectra of the two compounds differed from each other in the chemical shifts of the carbon atoms of the carboxyl group and of the C-1 of the benzoate moiety and of the C-1 of Glc (C-1'). From the signal in the ^{1}H NMR spectrum at 5.09 ppm (J = 7.3 Hz; H-1 of Glc) and the signals in the ^{13}C NMR spectrum at 101.7 ppm (C-1'), 125.7 ppm (C-1), and 169.6 ppm (COO), compound 1 was identified as the phenolic glucoside of 4HB, 4-*O*-(1-β-D-glucosyl)benzoic acid (Fig. 4, 1) (Klick and Herrmann, 1988; Tabata et al., 1988). The corresponding signals for compound 2 were found at 5.54 ppm for H-1 of Glc and at 94.6, 119.8, and 164.4 ppm for the three mentioned carbons. Therefore, compound 2 was identified as the ester glucoside of 4HB, 4HB 1-β-D-glucosyl ester (Fig. 4, 2) (Klick and Herrmann, 1988). An authentic reference substance for compound 1 was obtained, which gave identical spectroscopic data.

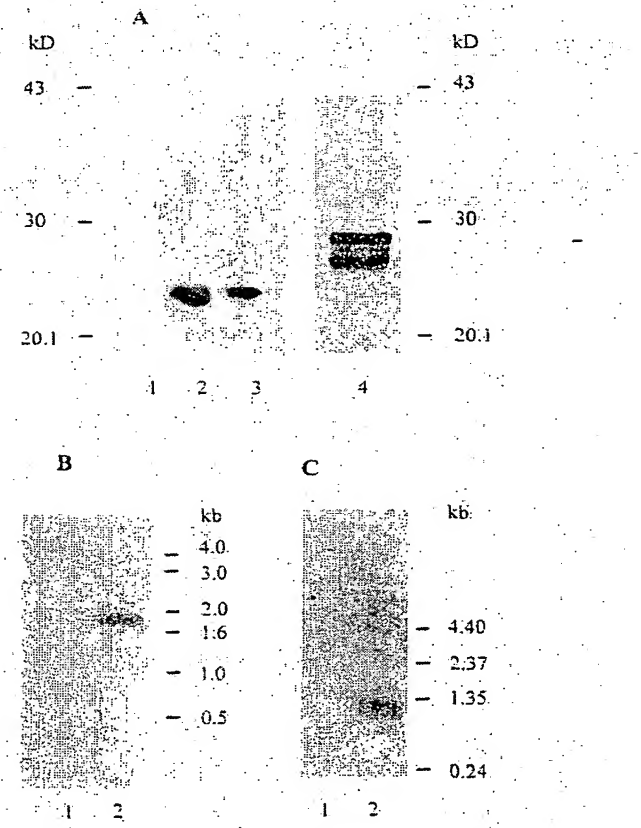


Figure 3. Analysis of tobacco transformed with the *ubiC* gene of *E. coli*. A, Immunoblots; B, genomic DNA gel blot; C, RNA gel blot. Lanes 1, Untransformed control (SR1); lanes 2, transgenic tobacco (transformant II); lanes 3, *E. coli* transformed with pUbiC; lanes 4, *E. coli* transformed with pTP-UbiC. For the genomic DNA gel blots, DNA was digested with *Hin*dIII and *Eco*RI and hybridized with a radioactive probe for the *ubiC* gene. A hybridization band with the expected size of 1.8 kb was detected in the transgenic tobacco (B, lane 2). RNA gel blots were hybridized with the same probe, and a band appeared in the transgenic tobacco at 1.1 kb as expected (C, lane 2). A specific antibody against UbiC was used for the immunoblot (Siebert et al., 1992). The UbiC protein expressed in transgenic plants (lane 2) has a calculated molecular mass of 19.8 kD after removal of the transit peptide; for the intact fusion with the transit peptide, 25.4 kD was calculated. *ubiC* fusion proteins expressed in *E.coli* are shown as a comparison: the LacZ-UbiC fusion (calculated molecular mass 20.5 kD) produced from pUbiC (lane 3); the LacZ-TP-UbiC fusion (calculated molecular mass 26.6 kD) produced from pTP-UbiC (lane 4).

Table II. *Fractionation of a crude enzyme extract from transgenic tobacco plants (transformant II)*

Cell-fractionation and enzyme assays were carried out as described in "Materials and Methods." Data represent mean values ± SD of two incubations.

Fraction	Alcohol Dehydrogenase	Shikimate Dehydrogenase	CPL
	nkat/mg protein	*nkat/mg protein*	*pkat/mg protein*
Crude extract	12.9 ± 0.3	2.65 ± 0.05	35.5 ± 0.5
Cytosol	18.2 ± 0.3	3.45 ± 0.05	32.0 ± 1.0
Organellar extract	3.0 ± 0.1	9.40 ± 0.03	68.0 ± 2.0

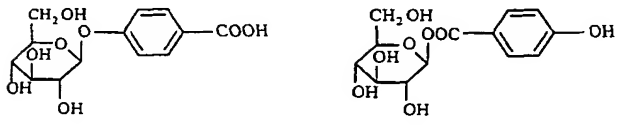
The total amount of 4HB derivatives, calculated as glucosides, was approximately 0.52% of dry weight. The phenolic glucoside **1** represented approximately 69%, and the ester glucoside **2** represented approximately 26% of this total amount of 4HB derivative. Free 4HB accounted for only 1.2% of the total, and another 1.7% was bound to the cell wall and could be released by acid or alkaline hydrolysis (see "Materials and Methods"). The ratio between phenolic and ester glucoside was essentially the same in all four transformants.

The conversion of free 4HB into glucosides may represent a detoxification mechanism of the free phenol, since in previous studies the same conjugates were also produced when various plant species were fed externally 4HB (Cooper-Driver et al., 1972; Klick and Herrmann, 1988).

Incorporation of [1,7-$^{13}C_2$]Shikimic Acid into 4-Hydroxybenzoate in the Transgenic Plants

The 4HB derivatives found in the *ubiC*-transformed plants were most likely formed because of CPL activity. However, the control plants were also expected to produce a certain amount of phenylpropanoid-derived 4HB for the biosynthesis of ubiquinones (Pennock and Threlfall, 1983). Furthermore, some plants and plant cell cultures have been reported to accumulate significant amounts of 4HB derivatives as secondary metabolites (Yazaki et al., 1986; Schnitzler et al., 1992), also derived from the phenylpropanoid pathway (Heide et al., 1989). To quantify to what extent the 4HB in our transgenic plants was a product of the artificially introduced CPL reaction or from the endogenous phenylpropanoid pathway, we carried out feeding experiments with [1,7-$^{13}C_2$]shikimic acid, analogous to published experiments with *E. coli* and *L. erythrorhizon* (Heide et al., 1989; Siebert et al., 1994). Feeding studies were carried out with cell-suspension cultures, which were established from the transgenic tobacco (transformant II). These cell cultures were shown to accumulate the same 4HB derivatives as in the intact transgenic plant (data not shown). After the feeding of the ^{13}C-labeled precursor the phenolic glucoside of 4HB was isolated, crystallized, subjected to ^{13}C NMR analysis (Fig. 5), and compared with the spectrum of an authentic reference substance. If two ^{13}C atoms in adjacent positions were incorporated from our [1,7-$^{13}C_2$]shikimate precursor (Fig. 1), each of the ^{13}C atoms would give a doublet rather than a singlet signal, because of the effect of ^{13}C-^{13}C coupling. The ^{13}C NMR spectrum in Figure 5 shows these doublets, in addition to the original peaks of the carboxyl group (peak a; 169.6 parts per million) and the neighboring ring position (peak b; 125.7 parts per million). This proves that both labeled carbons were incorporated (1.9% enrichment) into 4HB in the transgenic plants and that 4HB was formed as as a result of the introduced CPL reaction rather than by the endogenous phenylpropanoid pathway (Fig. 1). After calculating the integrals of the peaks and the accuracy of the measurement, we concluded that at least 95% of the 4HB, which was found as phenolic glucoside in the transgenic plants, was produced by the CPL reaction that had been introduced by genetic engineering.

The conversion of chorismate to 4HB presents a certain diversion from the natural flow of the shikimate path-



1 **2**

Figure 4. 4-Hydroxybenzoate derivatives accumulated in tobacco plants expressing the bacterial *ubiC* gene. **1**, 4-*O*-(1-β-D-glucosyl)-benzoic acid; **2**, 4HB 1-β-D-glucosyl ester.

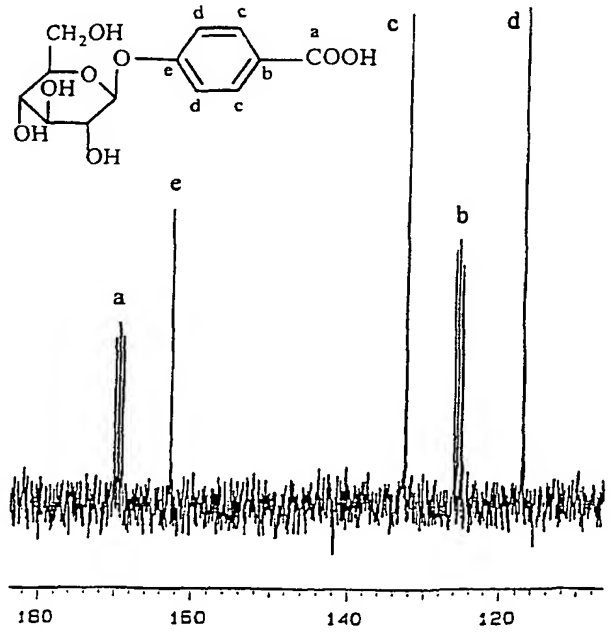


Figure 5. ^{13}C NMR spectrum of 4-*O*-(1-β-D-glucosyl)benzoic acid isolated from transgenic tobacco cell cultures after feeding of [1,7-$^{13}C_2$]shikimic acid. δ_{ppm} (CD_3OD, ^{13}C-depleted; 75 MHz); inverse-gated decoupling. Only the signals of the benzoic acid moiety are shown. Signals of Glc carbons appear between 62 and 102 parts per million. Peak a, 169.6 parts per million; peak b, 125.7 ppm; peak c, 132.7; peak d, 117.1; peak e, 162.8.

way, now producing a secondary metabolite instead of aromatic amino acids. However, the transgenic plants did not show visible phenotypic changes, suggesting that this pathway can be accommodated without seriously affecting other metabolic processes involving shikimate pathway intermediates.

In general, phenolics and especially 4HB esters show antimicrobial activity. In carrot cell cultures, as well as in alfalfa plants, the formation of 4HB can be elicited by treatment with fungal elicitors (Schnitzler et al., 1992; Cvrikova et al., 1993), suggesting a possible role of 4HB derivatives as phytoalexins. In addition, it has been reported that 4HB stimulates the production of pathogen-related proteins in *N. tabacum*, although to a considerably lower extent than salicylic acid (Abad et al., 1988). It will be interesting to test whether the expression of *ubiC* influences the pathogen resistance of plants.

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Metabolic engineering of medicinal plants: Transgenic *Atropa belladonna* with an improved alkaloid composition

(scopolamine/hyoscyamine 6β-hydroxylase)

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ABSTRACT **The tropane alkaloid scopolamine is a medicinally important anticholinergic drug present in several solanaceous plants. Hyoscyamine 6β-hydroxylase (EC 1.14.11.11) catalyzes the oxidative reactions in the biosynthetic pathway leading from hyoscyamine to scopolamine. We introduced the hydroxylase gene from *Hyoscyamus niger* under the control of the cauliflower mosaic virus 35S promoter into hyoscyamine-rich *Atropa belladonna* by the use of an *Agrobacterium*-mediated transformation system. A transgenic plant that constitutively and strongly expressed the transgene was selected, first by screening for kanamycin resistance and then by immunoscreening leaf samples with an antibody specific for the hydroxylase. In the primary transformant and its selfed progeny that inherited the transgene, the alkaloid contents of the leaf and stem were almost exclusively scopolamine. Such metabolically engineered plants should prove useful as breeding materials for obtaining improved medicinal components.**

The use of recombinant DNA technology for the manipulation of metabolic processes in cells promises to provide important contributions to basic science, agriculture, and medicine (1). Secondary metabolism is a particularly attractive target for the improvement of yields of desirable products, without markedly affecting basic cellular functions. The production of the antibiotic cephalosporin C by a fungal production strain has been improved by giving increased gene dosages of a rate-limiting enzyme (2). Several novel antibiotics have been produced by transferring all or part of their biosynthetic pathways to heterologous host microorganisms (1), as well as by targeted disruption of a biosynthesis gene (3). In plants, flavonoid pigments in ornamental flowers appear to be the most suitable for genetic modification because the flavonoid biosynthetic pathways and the genes involved are relatively well understood and because any changes in color and pigmentation patterns have potential commercial value (4). A great variety of pharmaceutical and antimicrobial compounds derived from plants also stand to benefit from yield improvement produced by genetic engineering, but a lack of understanding of the regulation of biosynthetic pathways and the general unavailability of cloned biosynthesis genes severely limit this approach at present. None of the introduced genes that are expected to function in target biosynthetic pathways have produced a considerable increase in the desired phytochemicals in transgenic plants (5–7).

The tropane alkaloids hyoscyamine (its racemic form being atropine) and scopolamine are used medicinally as anticholinergic agents that act on the parasympathetic nerve system. Because they differ in their actions on the central nervous system, currently there is a 10-fold higher commercial demand for scopolamine, in the *N*-butylbromide form, than there is for hyoscyamine and atropine combined. Several solanaceous species have been used as the commercial sources of these alkaloids, but the scopolamine contents in these plants often are much lower than those of hyoscyamine (8). For this reason there has been long-standing interest in increasing the scopolamine contents of cultivated medicinal plants. Naturally occurring and artificial interspecific hybrids of *Duboisia* have high scopolamine contents and are cultivated as a commercial source of scopolamine in Australia and other countries (9, 10). Anther culture combined with conventional interspecific hybridization also has been used to breed high scopolamine-containing plants in the genera *Datura* (11) and *Hyoscyamus* (12), but without much success.

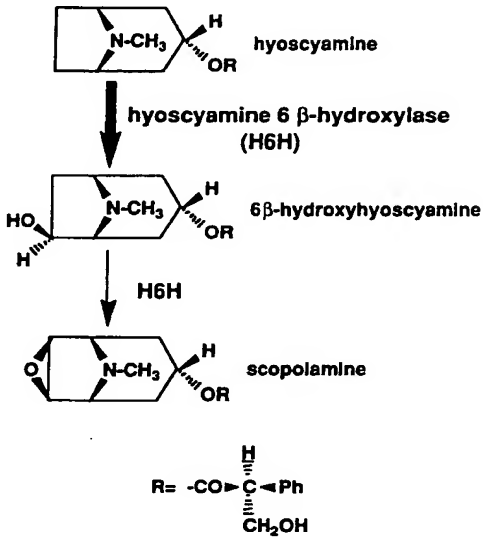

FIG. 1. Biosynthetic pathway from hyoscyamine to scopolamine. Scopolamine is formed from hyoscyamine via 6β-hydroxyhyoscyamine. H6H catalyzes the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine, as well as the epoxidation of 6β-hydroxyhyoscyamine to scopolamine.

Scopolamine is formed from hyoscyamine via 6β-hydroxyhyoscyamine (Fig. 1). Hyoscyamine 6β-hydroxylase (H6H; EC 1.14.11.11) catalyzes the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine, as well as the epoxidation of 6β-hydroxyhyoscyamine to scopolamine (13–15). Although the epoxidation activity of H6H is much lower than its hydroxylation activity, indirect evidence suggests that the epoxidation reaction may not be a limiting step *in planta*. 6β-Hydroxyhyoscyamine usually does not accumulate in scopolamine-producing plants (16). Moreover, a rough cor-

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Abbreviations: H6H, hyoscyamine 6β-hydroxylase; CaMV, cauliflower mosaic virus.

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relation has been found between H6H activity and the ratio of scopolamine to hyoscyamine in scopolamine-producing cultured roots (16). H6H therefore is a promising target enzyme which, if expressed strongly in hyoscyamine-accumulating plants, would result in increased scopolamine contents in the transformants.

Recently, an H6H cDNA clone was obtained from *Hyoscyamus niger* (17). We have introduced the H6H gene into, and expressed it in, *Atropa belladonna*, a typical hyoscyamine-rich plant. In the transformed plants conversion of hyoscyamine to scopolamine was highly efficient, evidence that the metabolic engineering of medicinal plants for the production of better pharmaceutical constituents is feasible.

MATERIALS AND METHODS

H6H Expression Vector. The *Xho* I fragment of the H6H cDNA insert was isolated from pBHH1 (17), filled-in with Klenow DNA polymerase, and ligated to a *Sal* I linker. The resulting DNA fragment was subcloned in pCaMVCN (Pharmacia) between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase terminator. The chimeric 35S–H6H gene then was excised as an *Xba* I fragment and subcloned in plant expression vector pGA482 (Pharmacia); the resulting binary vector is referred to as pHY8.

Plant Transformation. Seeds of *A. belladonna* were obtained from the Tsukuba Medicinal Plant Research Station (Ibaraki, Japan). The binary vectors (pHY8 and pGA482) were transferred to *Agrobacterium tumefaciens* LBA4404 by the direct transfer method (18). Exconjugants were used to transform belladonna leaf explants, basically as described for tobacco leaf disk transformation (18). All the leaf disks used were prepared from young leaves of a single seedling grown *in vitro*. After calli had formed on B5 agar medium (19) containing 10 μM 1-naphthaleneacetic acid and 1 μM 6-benzyladenine, shoots were induced on medium with 0.1 μM 1-naphthaleneacetic acid and 10 μM 6-benzyladenine and then transferred to hormone-free medium for root formation. All the culture media contained kanamycin at 500 μg/ml, carbenicillin at 500 μg/ml, and cefotaxime at 250 μg/ml. A total of 27 putative transformants that showed kanamycin resistance were screened for the expression of the H6H polypeptide by immunoblot analysis of the leaf samples. One plantlet that expressed the H6H polypeptide strongly in its leaves was selected and propagated from cuttings. Three plants propagated from the single primary transformant were planted in soil, then placed in a growth chamber, and grown at 20°C with a 14 hr/10 hr day/night regime and 80% humidity. Kanamycin-resistant plants regenerated from leaf disks treated with the LBA4404 strain harboring the empty vector pGA482 were the controls. Wild-type plants were grown in the same growth chamber. The T_0 primary transformant was self-pollinated, after which its seeds were collected. T_1 progeny were grown under the same conditions.

DNA Blot Analysis. DNA was isolated from belladonna leaves by the cetyltrimethylammonium bromide procedure (20). Southern hybridization was done as described (21). Genomic DNA was digested with *Bam*HI, then electrophoresed in a 0.7% (wt/vol) agarose gel, and blotted onto GeneScreen*Plus* (DuPont). DNA gel blots were probed with a 1.4-kilobase-pair (kbp) *Xho* I fragment from pBHH1 that contained the H6H gene (17) or with a 2-kbp *Bam*HI–*Hin*dIII fragment from pGA482 that contained the neomycin phosphotransferase II gene. The probes were labeled by random priming to a specific activity of $\approx 3 \times 10^7$ Bq/μg of DNA. The filters were hybridized with probe in 1.08 M sodium chloride/6 mM sodium phosphate, pH 6.8/6 mM EDTA/0.5% SDS/50% formamide containing salmon sperm DNA (100 μg/ml) at 42°C for 24 hr. The blots were washed in 0.3 M sodium chloride/0.03 M sodium citrate, pH 7.0/1% SDS at 65°C.

Immunoblot Analysis. Leaves, stems, main roots, and branch roots were collected, frozen with liquid nitrogen, and homogenized. The homogenate was suspended in 100 mM phosphate buffer, pH 7.5/3 mM dithiothreitol and centrifuged at 13,000 × *g* for 20 min. The supernatant was precipitated with ammonium sulfate of 80% saturation. The precipitate obtained after centrifugation was dissolved in 50 mM Tris·HCl, pH 7.8/1 mM dithiothreitol and then desalted on a PD-10 column (Pharmacia).

The crude cell extracts (30 μg of protein per lane) were subjected to SDS/PAGE in a 12.5% separating gel (22), and the separated proteins were transferred electrophorectically to Immobilon (Millipore). Immunoblotting was done as described (23) with the anti-H6H monoclonal antibody mAb5.

Enzyme Assay. Gas–liquid chromatography was used to assay the crude cell extracts for H6H enzyme activity by measuring the formation of 6β-hydroxyhyoscyamine from hyoscyamine (16).

Alkaloid Analysis. Tropane alkaloids were extracted from plant tissues and then purified and quantified by gas–liquid chromatography, as described (24). Alkaloids were identified by comparing their mass spectra with those of authentic samples (25).

RESULTS

Transfer and Expression of the *H. niger* H6H Gene in *A. belladonna*. Tropane alkaloids, including hyoscyamine, are synthesized mainly in the root, after which they are translocated to the leaf (26). Ectopic expression of the H6H gene introduced into tissues other than at the site of biosynthesis may give the H6H enzyme access to its alkaloid substrates, hyoscyamine and 6β-hydroxyhyoscyamine, during the translocation and storage of these alkaloids in the aerial plant parts. We therefore used the CaMV 35S promoter to drive the expression of the H6H gene from *H. niger* in a wide variety of cell types. The chimeric 35S–H6H gene was introduced by a leaf-disk transformation system into *A. belladonna*, which accumulates hyoscyamine as its main alkaloid. A total of 27 kanamycin-resistant primary transformants were screened by immunoblot analysis for the expression of the H6H polypeptide in the leaf.

One transformant that expressed H6H particularly strongly was chosen, and after it had grown to maturity it was self-pollinated to give T_1 progeny. The primary T_0 transformant and its 10 randomly selected T_1 progeny, as well as the wild-type belladonna, were analyzed for the presence of the H6H gene by Southern hybridization of *Bam*HI-digested genomic DNA. An endogenous belladonna H6H gene was detected as an ≈10-kbp *Bam*HI fragment in all the samples (Fig. 2). The T_0 plant contained three copies of the introduced 35S–H6H chimeric gene as shown by three hybridizing bands at approximately 18, 20, and 23 kbp. These hybridizing bands segregated, apparently at random, in the T_1 progeny. Similar results were obtained when a neomycin phosphotransferase II gene was the probe (data not shown).

Next, four plant parts (leaf, stem, main root, and branch root) were analyzed by immunoblotting and by enzyme assays (Fig. 3). In the T_0 (data not shown) and T_1 plants, there was strong expression of the H6H polypeptide and H6H enzyme activity in all the parts. In wild-type belladonna, however, weak expressions of the polypeptide and enzyme activity were found only in the branch root. Transgenic belladonna plants that constitutively expressed *H. niger* H6H showed no obvious differences in growth and development when compared with the control and wild-type plants.

Alkaloid Analysis. The content and composition of tropane alkaloids are known to vary considerably during plant de-

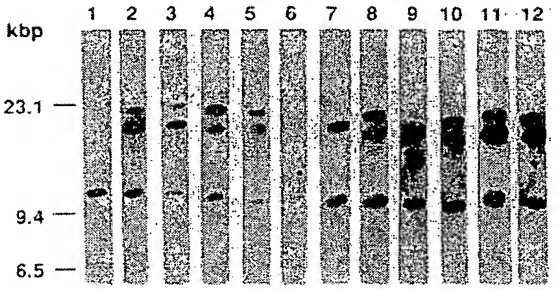

FIG. 2. Southern blot analysis of *A. belladonna* DNA digested with *Bam*HI. Lanes: 1, wild-type belladonna; 2, primary transformant T_0; 3, plant T_1-5; 4, plant T_1-11; 5, plant T_1-12; 6, plant T_1-13; 7, plant T_1-20; 8, plant T_1-24; 9, plant T_1-25; 10, plant T_1-26; 11, plant T_1-27; 12, plant T_1-28. T_1 plants are progeny of self-fertilized T_0. The blot was probed with the random-primed cDNA insert of pBHH1. Positions of size markers (*Hin*dIII-digested λ phage DNA) are shown at left.

velopment (27, 28). We found that the scopolamine content of *A. belladonna* was relatively high in the seedling and vegetative stages but progressively decreased toward the flowering stage, at which time total alkaloid content had considerably increased and hyoscyamine was the predominant alkaloid. We first analyzed the tropane alkaloids in the leaves of 3-month-old transformants during the preflowering stage (Table 1). The T_0 plant and 73 of the 74 T_1 plants showed a high-scopolamine phenotype, in which scopolamine made up >70% of the total alkaloid content. One T_1 plant (T_1-57) had a low percentage (35%) of scopolamine in its leaf alkaloids, which was comparable to the scopolamine percentages found in the wild-type and control plants during the corresponding developmental stages. This segregation ratio for the high-scopolamine phenotype in the T_1 progeny is expected from the estimation that three copies of the transgene are integrated in the T_0 transformant.

We next analyzed in detail the alkaloids present in mature plants after seeds had formed (Fig. 4). During this stage, >92% (average, 97%) of the total alkaloid contents of the leaf, stem, and main root of the wild-type and control plants was hyoscyamine. The branch roots of these plants contained relatively high amounts of scopolamine, which reflects the

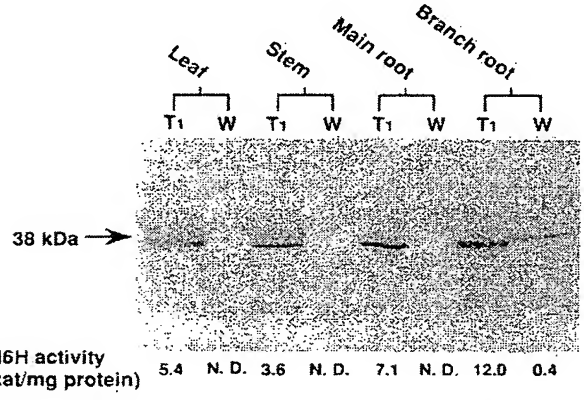

FIG. 3. Western blot analysis and H6H enzyme activity of *A. belladonna*. The immunoblot was probed with an H6H-specific antibody, mAb5. The position of the H6H polypeptide (38 kDa) is indicated. W, wild-type plant. T_1, pooled sample from transgenic plants T_1-14, -24, and -25. When 3-month-old plants were analyzed, >80% of total alkaloids in the leaves of these T_1 plants was scopolamine, as shown in Table 1. N.D., not detected.

Table 1. Distribution of scopolamine phenotypes among different 3-month-old belladonna plants

	Scopolamine percentage in total leaf alkaloids*					
	0–20	20–40	40–60	60–80	80–100	Total†
Wild-type and control plants	7					7
Primary transformants					3	3
T_1 progeny		1		1	72	74

*Scopolamine, hyoscyamine, and 6β-hydroxyhyoscyamine were identified in the leaf. In the plants with low scopolamine contents, hyoscyamine was the main alkaloid.
†Total number of plants analyzed.

endogenous H6H activity expressed in this organ. The alkaloid contents in the aerial parts of the T_0 transformant and five randomly selected T_1 progeny [all confirmed by Southern hybridization to contain at least one copy of the 35S–H6H transgene (Fig. 2)] were composed almost exclusively of scopolamine. In the main roots of some T_1 progeny, and particularly in the branch roots of the T_0 and T_1 transformants, hyoscyamine conversion was not as efficient as in the aerial parts. Possibly because of unintended subtle differences in the growth conditions under which the groups of plants were grown (sometimes successively in one growth chamber), the total alkaloid contents may have varied markedly among the genotypes, with total leaf alkaloids tending to be higher in the T_1 progeny than in the other plants. Further tests in the greenhouse and in the field are necessary to assess the long-term performance of these transgenic scopolamine-type belladonnas, especially with regard to the total yields of alkaloids.

DISCUSSION

Transfer of the H6H gene into, and its constitutive expression in, hyoscyamine-rich belladonna converted the herbaceous perennial to a chemotype pharmacologically more valuable. The change in the alkaloid composition in transgenic belladonna was remarkably efficient: scopolamine was almost the only alkaloid present in the aerial parts. This almost exclusive presence of scopolamine in medicinal plants cannot be achieved by conventional breeding methods. From such plants, pure scopolamine can be isolated by recrystallization of the total alkaloid fraction from leaf samples, instead of by the conventional differential extraction and chromatography of each component alkaloid. Although the primary transformant and the majority of its T_1 progeny received more than one copy of the chimeric 35S–H6H gene, alkaloid analysis of the T_1-13 and T_1-20 progeny plants showed that a single copy of the transgene was sufficient to create an all-scopolamine chemotype in the leaf.

The H6H purified from the cultured roots of *H. niger* (15, 16) catalyzed two consecutive reactions (6β-hydroxylation and 6,7-epoxidation) in the conversion of hyoscyamine to scopolamine, but epoxidase activity inherently was very weak (about 2–5% of the hydroxylase activity). Nevertheless, when 0.2 mM hyoscyamine and 6β-hydroxyhyoscyamine were fed to wild-type tobacco plants that lacked the H6H gene and to transgenic tobacco plants that constitutively expressed the 35S–H6H transgene, both alkaloids were efficiently converted to scopolamine in the leaves of the transgenic tobacco, but not in those of the wild-type tobacco (unpublished work). H6H therefore must be responsible for the high-scopolamine chemotype in transgenic belladonna plants and, probably, in naturally scopolamine-rich species such as *H. niger* and *Duboisia myoporoides* as well.

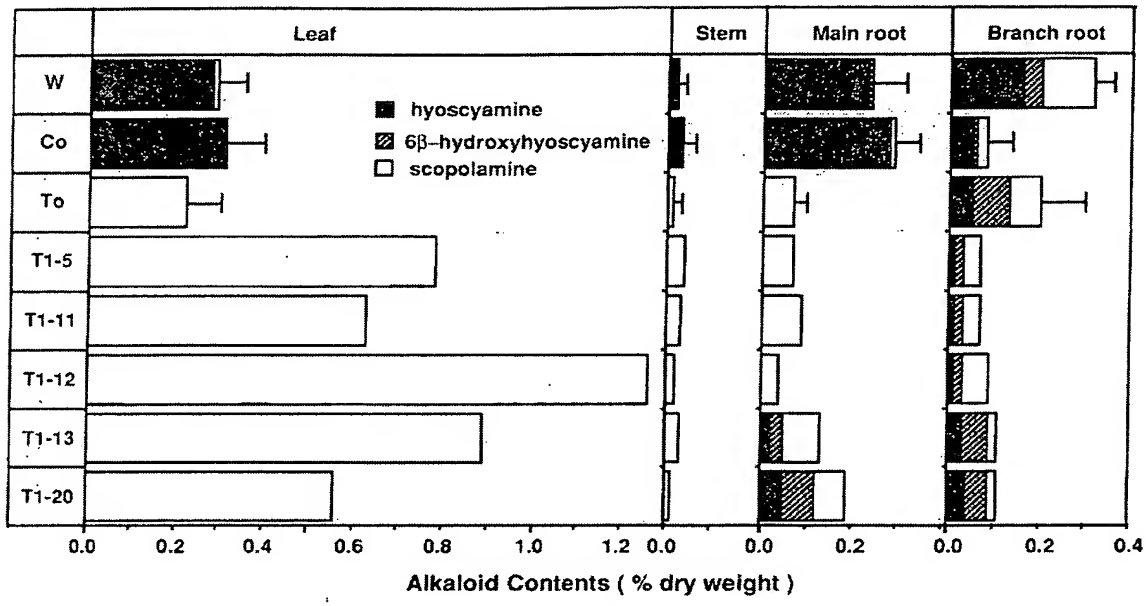

FIG. 4. Alkaloid contents in *A. belladonna*. W, wild-type plants; Co, control plants transformed with pGA482; T_0, transgenic plants transformed with pHY8; T_1, selfed progeny of T_0. After their seeds had been collected, the mature plants were harvested. All the T_0 and T_1 plants shown here had been confirmed to express the H6H polypeptide, at least in the leaves. Tropane alkaloid content was analyzed in the upper leaves, lower stem, main root, and branch root. Data for three independent plants each of W, Co, and T_0 were pooled and expressed as means ± SD.

In contrast to the highly efficient conversion of hyoscyamine to scopolamine in the aerial parts of the transgenic belladonna, the 6β-hydroxyhyoscyamine and scopolamine contents were enhanced only marginally in the branch roots. The H6H polypeptide and the enzyme activity were also expressed relatively strongly in the branch roots as well as in the aerial parts of the transgenic belladonna (Fig. 3). Although involvement of unidentified factors (e.g., novel enzymes) cannot be rigorously excluded, we suggest two alternative, nonexclusive possibilities for the difference in scopolamine content between aerial parts and roots. (*i*) Hyoscyamine may be synthesized in a restricted set of root cells in which the CaMV 35S promoter functions poorly. Specific localization of the endogenous H6H polypeptide in the pericycle cells of wild-type branch roots (23) indicates that its substrate hyoscyamine should be present, and may even be localized, in this cell type. The 35S promoter contains domain A (nucleotides −90 to +8), which drives the expression of the downstream gene in root tissue, including the pericycle (29). However, the strength of the entire 35S promoter in the pericycle cells, as compared with the other root cells, has not been reported. We analyzed the expression from the 35S promoter by using histochemical staining of the β-glucuronidase activity in another transgenic belladonna but found no evidence of poor expression of the reporter gene in the pericycle (data not shown).

(*ii*) Efficient conversion from hyoscyamine to scopolamine may occur during the translocation and storage of alkaloids. Classical reciprocal grafting experiments between alkaloid-producing and nonproducing solanaceous plants (26) showed that tropane alkaloids were synthesized mainly in the root and translocated to the aerial plant parts by a process also present in nonproducing plants. The feeding of labeled atropine (the racemic form of hyoscyamine) to steam-girdled belladonna plants indicated that tropane alkaloid translocation occurred through the xylem (T. Hartmann, personal communication). The alkaloids carried in the xylem sap ultimately are stored in various types of cells in the aerial parts, probably in the vacuole (30). The rate of alkaloid translocation in plants is not well understood but may be slow enough to allow the H6H expressed in the xylem or intervening tissues to act efficiently on alkaloids during translocation. The use of tissue-specific promoters to drive the expression of the H6H gene should provide more information about the tissues in which alkaloid conversion mainly takes place.

Although we used *A. belladonna* as the transgenic host, the metabolic engineering method reported here obviously is not limited to that species. Several medicinal plants that long have been known as rich sources of hyoscyamine (8) but that have been considered unattractive for commercial exploitation because of their low scopolamine contents may now become promising candidates as sources of scopolamine. Commercial cultivars of *Duboisia* hybrids that contain naturally high levels of scopolamine also may be improved by our method. Many of these solanaceous species are susceptible to *Agrobacterium* infection (31, 32), and they can be regenerated from tissue cultures to whole plants (32, 33). Metabolically engineered medicinal plants will complement conventional breeding efforts for the production of quantitatively and qualitatively improved pharmacological properties.

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Transgene-Mediated and Elicitor-Induced Perturbation of Metabolic Channeling at the Entry Point into the Phenylpropanoid Pathway

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^{3}H-L-Phenylalanine is incorporated into a range of phenylpropanoid compounds when fed to tobacco cell cultures. A significant proportion of ^{3}H-*trans*-cinnamic acid formed from ^{3}H-L-phenylalanine did not equilibrate with exogenous *trans*-cinnamic acid and therefore may be rapidly channeled through the cinnamate 4-hydroxylase (C4H) reaction to 4-coumaric acid. Such compartmentalization of *trans*-cinnamic acid was not observed after elicitation or in cell cultures constitutively expressing a bean phenylalanine ammonia–lyase (*PAL*) transgene. Channeling between PAL and C4H was confirmed in vitro in isolated microsomes from tobacco stems or cell suspension cultures. This channeling was strongly reduced in microsomes from stems or cell cultures of transgenic *PAL*-overexpressing plants or after elicitation of wild-type cell cultures. Protein gel blot analysis showed that tobacco PAL1 and bean PAL were localized in both soluble and microsomal fractions, whereas tobacco PAL2 was found only in the soluble fraction. We propose that metabolic channeling of *trans*-cinnamic acid requires the close association of specific forms of PAL with C4H on microsomal membranes.

INTRODUCTION

The phenylpropanoid pathway is involved in the biosynthesis of a wide variety of natural products from plants. Many of these products have important functions in plant development and in interactions of the plant with its environment (Hahlbrock and Grisebach, 1979; Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995). Many studies have addressed the transcriptional regulation of genes encoding enzymes of the phenylpropanoid pathway and subsequent changes in extractable enzyme activities in response to developmental and environmental cues (Cramer et al., 1985; Lawton and Lamb, 1987; Hahlbrock and Scheel, 1989). Far less attention has been paid to how the cell regulates flux into different end products of the pathway once all of the enzymatic machinery is assembled.

The first committed step in the biosynthesis of phenylpropanoid compounds is the conversion of L-phenylalanine (Phe) to *trans*-cinnamic acid by L-Phe ammonia–lyase (PAL; EC 4.3.1.5; Figure 1). PAL is a tetrameric enzyme whose subunits are encoded by a multigene family in most species that have been studied (Cramer et al., 1989; Nagai et al., 1994; Wanner et al., 1995; Fukasawa-Akada et al., 1996). *PAL* genes are transcriptionally activated after microbial infection or treatment of plant cells with microbial elicitors (Edwards et al., 1985; Lawton and Lamb, 1987). The second step in the phenylpropanoid pathway, the hydroxylation of *trans*-cinnamic acid to 4-coumaric acid, is catalyzed by a cytochrome P450 monooxygenase, cinnamic acid 4-hydroxylase (C4H; EC 1.14.13.11; Russell and Conn, 1967; Fahrendorf and Dixon, 1993; Teutsch et al., 1993). C4H is induced by light, elicitors, and wounding (Fahrendorf and Dixon, 1993; Buell and Somerville, 1995; Batard et al., 1997; Bell-Lelong et al., 1997), and its induction often is closely coordinated with PAL induction (Mizutani et al., 1997).

Enzymes of complex metabolic pathways may be present in the cell in arrays of consecutive, physically associated enzymes that are assembled on membranes or other physical structures (Subramanian et al., 1973; Margna and Margna, 1978; Cutler and Conn, 1981; Cutler et al., 1981; Srere, 1987). Such enzyme organization can result in the channeling of pathway intermediates without their release into general metabolic pools (Stafford, 1981; Hrazdina, 1992). Cytochrome P450 enzymes, such as C4H, are anchored to the external surface of the endoplasmic reticulum (Chapple, 1998), but PAL has been regarded generally as an operationally soluble enzyme. However, studies performed with microsomes isolated from potato (Czichi and Kindl, 1975) and cucumber cotyledons (Hrazdina and Wagner, 1985)

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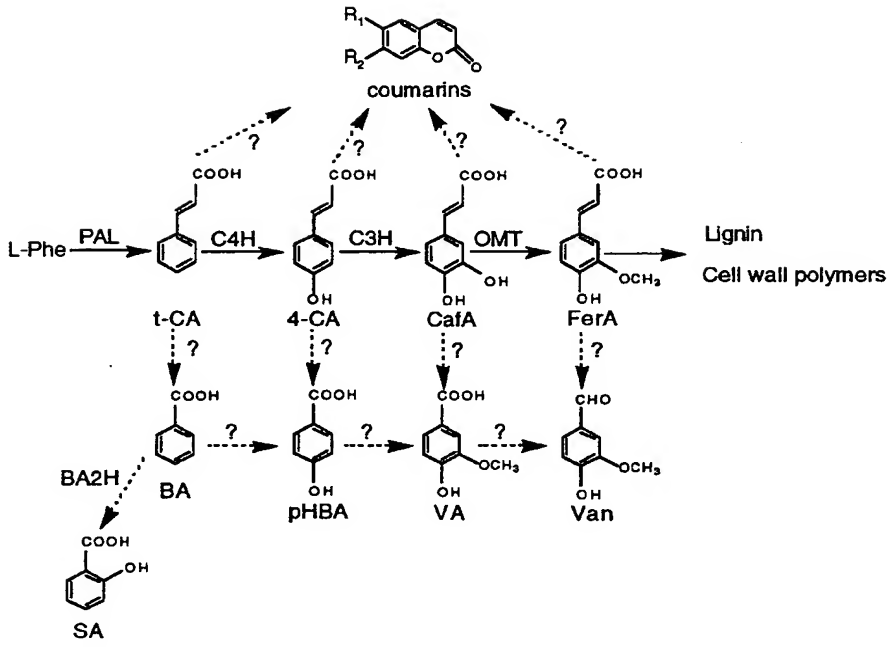

Figure 1. Biosynthesis of Phenolic Compounds in Tobacco.

L-Phe is deaminated by PAL to yield *trans*-cinnamic acid (t-CA), which is converted by C4H to 4-coumaric acid (4-CA). 4-Coumaric acid can be hydroxylated by coumarate 3-hydroxylase (C3H) to caffeic acid (CafA), which is acted upon by an *O*-methyltransferase (OMT) to yield ferulic acid (FerA). Salicylic acid (SA) and pHBA are formed via chain shortening of *trans*-cinnamic acid and 4-coumaric acid, respectively, although the specific mechanisms are not clear. Similarly, the exact biosynthetic origins of vanillin (Van), vanillic acid (VA), and coumarins (such as scopoletin in tobacco) have not been determined definitively; these pathways are indicated by dotted lines and question marks. Benzoic acid (BA) is most probably the precursor of salicylic acid, which is formed by the action of a benzoic acid 2-hydroxylase (BA2H). R_1 and R_2 are commonly substituted positions in coumarins. In scopoletin, $R_1 = OCH_3$ and $R_2 = OH$.

have suggested that PAL and C4H activities are colocalized on membranes of the endoplasmic reticulum. Furthermore, *trans*-cinnamic acid formed endogenously via the PAL reaction is a better substrate for C4H than externally added *trans*-cinnamic acid in in vitro assays. This finding has been interpreted as evidence for channeling of *trans*-cinnamic acid in the conversion of Phe to 4-coumaric acid (Czichi and Kindl, 1975, 1977; Hrazdina and Wagner, 1985; Hrazdina and Jensen, 1992).

To gain further insight into the phenomenon of metabolic channeling, it is necessary to develop a model system that allows for comparison of metabolic compartmentalization/channeling in vivo and in vitro. This system must be amenable to manipulation to increase or decrease various components of the potential channel, and molecular information on the gene products involved in channeling must be available. Tobacco represents such a system. We have generated a series of transgenic tobacco plants containing a heterologous bean *PAL2* gene, in which the levels of extractable PAL activity are increased or decreased when compared with wild-type plants (Elkind et al., 1990; Howles et al., 1996; Sewalt et al., 1997). These plants allow assessment of the effects of quantitative and qualitative changes in a component of a potential metabolic channel on the operation of that channel. Furthermore, it is now possible to design probes to distinguish between different members of the *PAL* gene family and therefore to address which forms of PAL may be associated with channeling.

Here, we present evidence, from in vivo and in vitro labeling experiments with ^{3}H-L-Phe and ^{14}C-*trans*-cinnamic acid, for metabolic channeling that involves coupling of PAL and C4H in tobacco stem tissue and cell suspension cultures. We demonstrate that specific forms of PAL are associated with tobacco microsomes and that microsomal association of a heterologous PAL enzyme in transgenic plants can perturb channeling. We also show that metabolic channeling is no longer measurable after activation of the phenylpropanoid pathway by elicitation.

RESULTS

Metabolic Compartmentalization of *trans*-Cinnamic Acid in Tobacco Cell Suspension Cultures

If a biosynthetic intermediate is channeled, it will not equilibrate freely with an externally added intermediate, and the endogenously formed and externally supplied compound will exist in the cell in different pools. In vivo labeling experiments can show the existence of different metabolic pools, although these pools may or may not result from metabolic channeling. Conversely, demonstration of a single pool of a metabolite that fully equilibrates with the externally added compound would constitute evidence against channeling. Therefore, we used tobacco cell suspension cultures to confirm the existence of more than one pool of *trans*-cinnamic acid in tobacco as a precursor to further studies on channeling between PAL and C4H that make use of transgenic plants and in vitro assays. When fed ^{3}H-L-Phe, such cultures accumulate a range of labeled phenolic compounds, including the conjugates of *trans*-cinnamic acid, 4-coumaric acid, caffeic acid, and ferulic acid, as shown in Figure 2A. These compounds are derived from Phe via *trans*-cinnamic acid and 4-coumaric acid, as shown in Figure 1.

If suspension-cultured tobacco cells possessed only a single pool of *trans*-cinnamic acid with which ^{3}H-*trans*-cinnamic acid formed by the PAL reaction from ^{3}H-Phe readily equilibrated, simultaneous feeding of ^{3}H-Phe and unlabeled *trans*-cinnamic acid would result in a lowering of the specific activity of tritium in the various phenylpropanoid compounds derived from *trans*-cinnamic acid when compared with the values obtained in the absence of unlabeled *trans*-cinnamic acid. At the end of the labeling period, the specific activity of reisolated *trans*-cinnamic acid would represent the minimum specific activity of the ^{3}H-*trans*-cinnamic acid precursor pool, assuming no metabolic compartmentalization, and the final specific activity of the various phenylpropanoid compounds would be equal to or lower than this value, depending on the sizes of their pools and the pools of the intermediates of the pathway during the period of labeling. Thus, assuming complete equilibration of endogenously formed ^{3}H-*trans*-cinnamic acid with endogenous unlabeled internal pools and externally applied unlabeled *trans*-cinnamic acid, the ratio of the specific activity of a particular product to the specific activity of the total extractable *trans*-cinnamic acid should be 1.0 or <1.0.

Figure 3 shows the results from a series of such isotope dilution experiments with tobacco cell cultures. In the absence of externally applied unlabeled *trans*-cinnamic acid, the product/precursor specific activity ratio was between 0.2 and 0.99 for 4-coumaric acid, caffeic acid, and ferulic acid (Figure 3A). Small but not statistically significant increases in the product/precursor specific activity ratios were observed for all compounds after simultaneous feeding of 10^{-5} M unlabeled *trans*-cinnamic acid and ^{3}H-Phe. However,

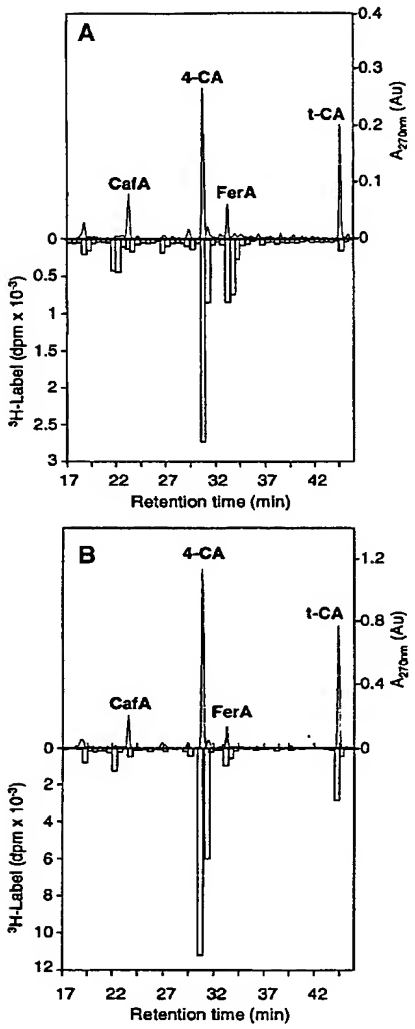

Figure 2. HPLC Traces Showing Incorporation of Tritium from ^{3}H-L-Phe into Phenylpropanoid Compounds in Tobacco Cell Suspension Cultures.

(A) Wild-type tobacco cell suspension cultures.

(B) Tobacco cell suspension cultures overexpressing PAL from the bean *PAL2* gene (OX434).

Cultures were treated simultaneously with 10^{-4} M ^{3}H-L-Phe and unlabeled *trans*-cinnamic acid (10^{-4} M). Cells were harvested 24 hr after the addition of the precursors. Extracts of soluble phenolics were treated with esterase and separated by HPLC. Fractions were collected, and radioactivity in *trans*-cinnamic acid (t-CA), 4-coumaric acid (4-CA), caffeic acid (CafA), and ferulic acid (FerA) was determined by liquid scintillation counting. Au, absorbance units.

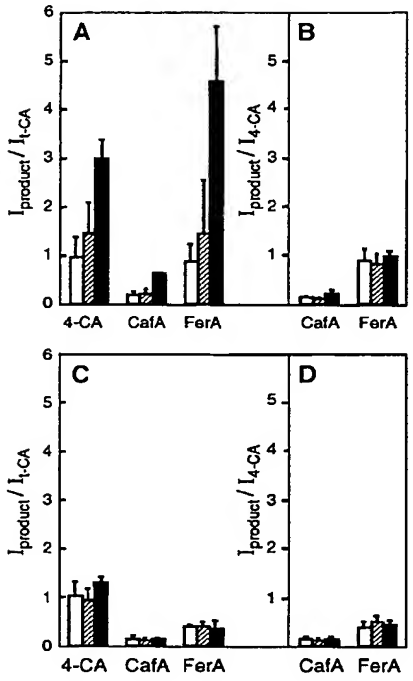

Figure 3. In Vivo Labeling Experiments Demonstrate Metabolic Compartmentalization of *trans*-Cinnamic Acid in Tobacco Cell Suspension Cultures.

Results are expressed as the ratio of incorporation of ^{3}H–L-Phe (determined as specific activity) in a particular phenolic product ($I_{product}$) to that in reextracted *trans*-cinnamic acid ($I_{t\text{-}CA}$) or 4-coumaric acid ($I_{4\text{-}CA}$). Effects of the addition of unlabeled *trans*-cinnamic acid and 4-coumaric acid on the ratio of $I_{product}$ to $I_{t\text{-}CA}$ and to $I_{4\text{-}CA}$ are shown. Results shown are the means ±SD of two independent experiments. Compounds were extracted 24 hr after treatment.

(A) Effects of the addition of unlabeled *trans*-cinnamic acid on the ratio of $I_{product}$ to $I_{t\text{-}CA}$ in wild-type tobacco cell suspension cultures.

(B) Effects of the addition of unlabeled 4-coumaric acid on the ratio of $I_{product}$ to $I_{4\text{-}CA}$ in wild-type tobacco cell suspension cultures.

(C) Effects of the addition of unlabeled *trans*-cinnamic acid on the ratio of $I_{product}$ to $I_{t\text{-}CA}$ in *PAL*-overexpressing tobacco cell suspension cultures.

(D) Effects of the addition of unlabeled 4-coumaric acid on the ratio of $I_{product}$ to $I_{4\text{-}CA}$ in *PAL*-overexpressing tobacco cell suspension cultures.

Open bars, no unlabeled *trans*-cinnamic acid or 4-coumaric acid; hatched bars, 10^{-5} M *trans*-cinnamic acid or 4-coumaric acid; filled bars, 10^{-4} M *trans*-cinnamic acid or 4-coumaric acid. Extracts of soluble phenolics were treated with esterase and separated by HPLC. Fractions were collected and radioactivity determined by liquid scintillation counting. CafA, caffeic acid; FerA, ferulic acid; 4-CA, 4-coumaric acid.

much larger increases (up to fivefold for ferulic acid) were obtained after feeding of 10^{-4} M unlabeled *trans*-cinnamic acid, due to a very large decrease in the specific activity of *trans*-cinnamic acid, whereas the specific activity of reisolated 4-coumaric acid remained in the same range as that of the downstream products. In a similar experiment in which unlabeled 4-coumaric acid was fed simultaneously with ^{3}H-Phe, no increase in the ratios of product/4-coumaric acid precursor specific activities were observed (Figure 3B). The same results were obtained when incorporation of the coumarin scopoletin or ^{3}H into *p*-hydroxybenzoic acid (pHBA) was measured in the presence or absence of unlabeled *trans*-cinnamic acid or 4-coumaric acid (data not shown). These results indicate that externally added unlabeled *trans*-cinnamic acid does not dilute isotope incorporation from ^{3}H-Phe into phenylpropanoid compounds in tobacco cell cultures, whereas externally added unlabeled 4-coumaric acid does, suggesting a specific metabolic compartmentalization of *trans*-cinnamic acid. This does not directly prove channeling or necessarily indicate the presence of more than one endogenous pool of *trans*-cinnamic acid in the absence of an externally fed compound.

PAL-overexpressing tobacco lines, resulting from constitutive expression of a bean *PAL2* gene, overproduce phenylpropanoid compounds (Howles et al., 1996), whereas underexpressing lines, resulting from epigenetic gene silencing (Elkind et al., 1990; Bate et al., 1994), have reduced levels of phenylpropanoid compounds. We initiated callus and then cell suspension cultures from *PAL*-overexpressing transgenic tobacco; constitutive PAL activities in the cell suspension cultures were at least threefold higher than in comparable cultures derived from wild-type plants. We then repeated the above-mentioned in vivo labeling experiments to determine whether upregulation of PAL activity affects metabolic compartmentalization of *trans*-cinnamic acid.

Figure 2B shows the pattern of phenylpropanoid compounds and the incorporation of tritium from ^{3}H–L-Phe into these compounds in *PAL*-overexpressing cultures. As shown in Figures 3C and 3D, expression of the bean *PAL2* transgene results in a loss of metabolic compartmentalization of *trans*-cinnamic acid. Thus, in contrast to the situation with wild-type cultures (Figure 3A), the addition of unlabeled *trans*-cinnamic acid had no effect on the ratios of product/precursor specific activities for the various phenylpropanoid compounds analyzed (Figure 3C). This indicates full equilibration of endogenously formed ^{3}H-*trans*-cinnamic acid with the pool of externally added compound in *PAL*-overexpressing cultures. As occurs with the wild-type cultures (Figure 3B), unlabeled 4-coumaric acid had no effect on the product/precursor specific activity ratio in *PAL*-overexpressing cultures (Figure 3D). Again, the same results were obtained when incorporation of ^{3}H into scopoletin or pHBA was measured in the presence or absence of unlabeled *trans*-cinnamic acid or 4-coumaric acid (data not shown).

In *PAL*-overexpressing transgenic tobacco plants, constitutive PAL activity is increased without a corresponding in-

crease in constitutive C4H activity (Howles et al., 1999). In contrast, elicitation induces a coordinated increase in both activities (Howles et al., 1999). We repeated the above-mentioned in vivo labeling experiments with suspension cultures of wild-type tobacco that had been exposed to a yeast elicitor for 6 hr before the addition of an isotopic label. The addition of unlabeled *trans*-cinnamic acid had little effect on the ratios of product/precursor specific activities in the various phenylpropanoid compounds analyzed (Figure 4C), in contrast to the situation with unelicited cultures shown in Figure 4A. This indicates full equilibration of endogenously formed ^{3}H-*trans*-cinnamic acid with the pool of externally added compound in elicited cultures. As occurs with the unelicited cultures (Figure 4B), unlabeled 4-coumaric acid had no effect on the product/precursor specific activity ratio in elicited cultures (Figure 4D). These results indicate that coordinated activation of the phenylpropanoid pathway as a result of elicitation results in a loss of the metabolic compartmentalization of *trans*-cinnamic acid observed in unelicited cultures.

Changes in Phenolic Metabolites as a Result of Elicitation or *PAL* Overexpression

To determine whether metabolic compartmentalization of *trans*-cinnamic acid is associated with accumulation of specific products of phenylpropanoid biosynthesis, we compared the patterns and amounts of phenolic metabolites resolved by HPLC from extracts of wild-type and *PAL*-overexpressing cell suspension cultures by using the cell samples analyzed in Figures 2 and 3. As shown in Table 1, there was a twofold increase in *trans*-cinnamic acid levels in *PAL*-overexpressing cultures compared with wild-type cell cultures, presumably due to the higher PAL activity in these cells. *PAL* overexpression led to a very strong accumulation (up to 10-fold) of a compound that we tentatively identified as a vanillin derivative on the basis of its UV spectrum and chromatographic properties (λ_{max} of 204, 228, 276, and 304 nm; λ_{min} of 216 and 246 nm; 97% spectral identity to vanillin; HPLC retention time of 31.4 min, eluting at 24% acetonitrile) as well as a twofold increase in scopoletin. No significant difference in the accumulation of 4-coumaric acid, ferulic acid, pHBA, salicylic acid, or vanillic acid could be observed in the two different cell lines, although caffeic acid levels were reduced by 50% in the *PAL*-overexpressing cells. The addition of *trans*-cinnamic acid resulted in higher levels of all of the above-mentioned metabolites, except for salicylic acid, in *PAL*-overexpressing cells when compared with wild-type cells. This indicates that the perturbation of channeling as a result of *PAL* overexpression leads to increased metabolism of exogenous *trans*-cinnamic acid.

Surprisingly, elicitation of wild-type cultures resulted in lower levels of most of the extractable phenolics, as shown in Table 2. The addition of 10^{-4} M *trans*-cinnamic acid led to threefold and 5.5-fold increases in 4-coumaric acid levels and 20-fold and 10-fold increases in *trans*-cinnamic acid levels in unelicited and elicited cells, respectively, but the total amounts of 4-coumaric acid, caffeic acid, ferulic acid, and scopoletin were ~50% lower in the elicited cells. This may be due to more rapid metabolism of these compounds and

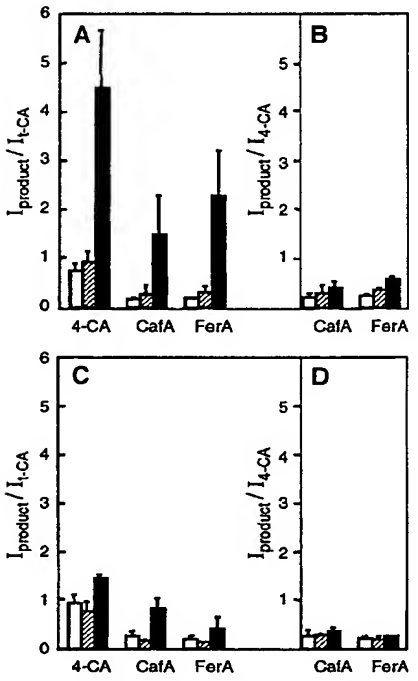

Figure 4. Effects of Elicitation on Metabolic Compartmentalization of *trans*-Cinnamic Acid in Tobacco Cell Suspension Cultures.

Results show the ratio of the $I_{product}$ to $I_{t\text{-}CA}$ and to $I_{4\text{-}CA}$ (see legend to Figure 3) in a particular phenolic compound in cells with or without elicitation. The experiment was conducted in the same way as described in the legend to Figure 3, except that 6 hr before the addition of ^{3}H-L-Phe and unlabeled *trans*-cinnamic acid or 4-coumaric acid, cells were treated either with water or the yeast elicitor, and cells were incubated with the precursors for only 6 hr before being harvested.
(A) Effects of the addition of unlabeled *trans*-cinnamic acid on the ratio of $I_{product}$ to $I_{t\text{-}CA}$ in unelicited tobacco cell suspension cultures.
(B) Effects of the addition of unlabeled 4-coumaric acid on the ratio of $I_{product}$ to $I_{4\text{-}CA}$ in unelicited tobacco cell suspension cultures.
(C) Effects of the addition of unlabeled *trans*-cinnamic acid on the ratio of $I_{product}$ to $I_{t\text{-}CA}$ in elicited tobacco cell suspension cultures.
(D) Effects of the addition of unlabeled 4-coumaric acid on the ratio of $I_{product}$ to $I_{4\text{-}CA}$ in elicited tobacco cell suspension cultures.
Open bars, no unlabeled cinnamic acids; hatched bars, 10^{-5} M *trans*-cinnamic acid or 4-coumaric acid; filled bars, 10^{-4} M *trans*-cinnamic acid or 4-coumaric acid. Results shown are the mean ±SD of two independent experiments. CafA, caffeic acid; FerA, ferulic acid; 4-CA, 4-coumaric acid.

Table 1. Effects of *PAL* Overexpression on Phenolic Compounds in Tobacco Cell Suspension Cultures

Unlabeled Precursor	Plant Line[a]	t-CA[b]	4-CA[b]	CafA[b]	FerA[b]	pHBA[b]	Scop[b]	SA[b]	VA[b]	Van-D[b]
None	WT	5	93	103	42	10	92	110	159	19
None	OX	12	109	52	56	8	185	73	182	209
10^{-4} M t-CA	WT	60	227	136	64	18	123	137	164	90
10^{-4} M t-CA	OX	170	618	201	219	18	478	139	263	426

[a] Soluble phenolic compounds were isolated from wild-type (WT) and *PAL*-overexpressing (OX) tobacco cell suspension cultures 24 hr after feeding with ^{3}H-L-Phe (10^{-4} M) in the presence or absence of unlabeled *trans*-cinnamic acid (t-CA).

[b] Amounts of phenolic compounds, quantified after enzymatic hydrolysis, are given as nanomoles per gram fresh weight. The compounds are *trans*-cinnamic acid, 4-coumaric acid (4-CA), caffeic acid (CafA), ferulic acid (FerA), pHBA, scopoletin (Scop), salicylic acid (SA), vanillic acid (VA), and vanillin derivative (Van-D). The experiments were conducted as described in the legend to Figure 3, and a duplicate experiment gave essentially the same results.

their subsequent deposition in the insoluble cell wall fraction. No significant amounts of these compounds could be detected in the culture medium or in the soluble cell wall fraction (data not shown).

Channeling of *trans*-Cinnamic Acid in Tobacco Stem Microsomes

Plant microsomes can convert L-Phe to 4-coumaric acid, which is not metabolized further to any significant degree (Czichi and Kindl, 1975, 1977; Hrazdina and Wagner, 1985). The value of the coupling factor, as defined by Czichi and Kindl (1977) and Kindl (1979), is a rigorous criterion for the coupling of enzymatic reactions in vitro. The coupling factor is used to compare the ratios of tritium to carbon-14 in the product (in this case, 4-coumaric acid) with the ratios of tritium to carbon-14 in the intermediate (in this case, *trans*-cinnamic acid) in dual labeling experiments. It thereby gives an estimation of the level of coupling between consecutive enzymes. The use of the ^{3}H-labeled primary substrate (L-Phe) and ^{14}C-labeled secondary substrate (*trans*-cinnamic acid) by enzymes located on isolated microsomes will result in the formation of labeled 4-coumaric acid with a specific tritium/carbon-14 ratio. If the value of the ratio in the product is higher than the respective value in the reisolated intermediate (i.e., a coupling factor >1.0), the bound or channeled form of the intermediate does not freely exchange with the external pool of the intermediate, indicating channeling between L-Phe and 4-coumaric acid.

We initially chose tobacco stem tissue for in vitro channeling assays because of the high levels of PAL and C4H activities in this tissue associated with lignification (Sewalt et al., 1997). We first confirmed the presence of PAL activity in microsomes isolated from tobacco stem tissue. The total activity of PAL in washed microsomes isolated from wild-type tobacco plants amounted to 5 to 10% of the total activity of PAL in the soluble enzyme fraction. As shown in Figure 5A, the specific activities of microsomal PAL were between 30 and 40% of the specific activities of soluble PAL. Microsomal PAL is relatively tightly associated with the microsomes and is not simply cytoplasmic contamination, as shown below.

Microsomal fractions were incubated simultaneously with ^{3}H-L-Phe and ^{14}C-*trans*-cinnamic acid, and the ratios of tritium to carbon-14 in reisolated *trans*-cinnamic acid and in 4-coumaric acid were compared. Coupling factors were between 5 and 11 in microsomes from wild-type plants,

Table 2. Effects of Elicitation on Phenolic Compounds in Tobacco Cell Suspension Cultures

Unlabeled Precursor	Treatment[a]	t-CA[b]	4-CA[b]	CafA[b]	FerA[b]	pHBA[b]	Scop[b]	SA[b]	VA[b]	Van-D[b]
None	Con	4	64	47	81	6	284	114	413	76
None	Eli	7	15	24	41	3	105	107	404	116
10^{-4} M t-CA	Con	86	195	44	101	7	372	97	476	118
10^{-4} M t-CA	Eli	69	79	23	48	7	164	130	357	95

[a] Soluble phenolic compounds were isolated from unelicited (Con) and elicited (Eli) tobacco cell suspension cultures after feeding with ^{3}H-L-Phe (10^{-4} M) in the presence or absence of unlabeled *trans*-cinnamic acid (t-CA).

[b] Amounts of phenolic compounds, quantified after enzymatic hydrolysis, are given as nanomoles per gram fresh weight. The compounds are *trans*-cinnamic acid, 4-coumaric acid (4-CA), caffeic acid (CafA), ferulic acid (FerA), pHBA, scopoletin (Scop), salicylic acid (SA), vanillic acid (VA), and vanillin derivative (Van-D). The experiments were conducted as described in the legend to Figure 4, with the compounds being isolated 12 hr after elicitation. A duplicate experiment gave essentially the same results.

suggesting significant channeling between PAL and C4H (Figure 5B). When soluble PAL from the cytoplasmic supernatant was added to the channeling assays, the ratio of tritium to carbon-14 in 4-coumaric acid remained almost the same, but the tritium/carbon-14 ratio in the reisolated *trans*-cinnamic acid intermediate was strongly increased, resulting in reduced coupling factors, as shown in Figure 5B. This is presumably due to an excess of ^{3}H-*trans*-cinnamic acid formed from ^{3}H-L-Phe, which is not converted to ^{3}H-4-coumaric acid because of the preference of the channeled system for ^{3}H-*trans*-cinnamic acid originating via the microsomal PAL reaction.

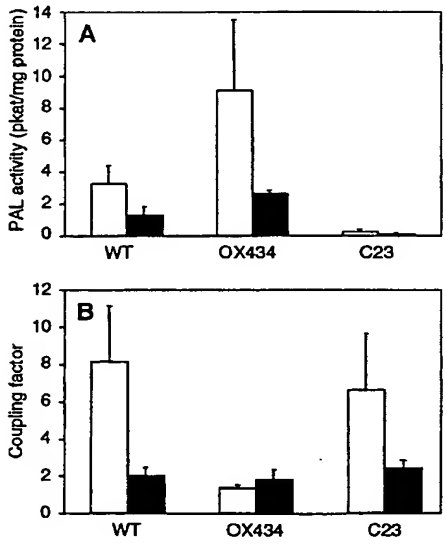

Figure 5. Specific Activities of PAL in the Soluble and Microsomal Protein Fractions and Metabolic Channeling between PAL and C4H in Microsomes from Stems of Wild-Type and Transgenic Tobacco Plants.

Plants were wild-type (WT), *PAL* overexpressers (OX434), or gene-silenced *PAL* underexpressers (C23).

(A) Open bars, soluble (105,000*g* supernatant) PAL activity; filled bars, microsomal (105,000*g* pellet) PAL activity. Total activities of microsomal PAL were 5 to 10% of total soluble PAL activity. pkat, picokatals.

(B) Washed and resuspended microsomes (250 to 500 μg of protein per assay) were incubated simultaneously with 500 nmol ^{3}H-L-Phe (5 nCi/nmol) and 30 nmol ^{14}C-*trans*-cinnamic acid (2 nCi/nmol). The coupling factor is defined as the tritium/carbon-14 ratio in the product, 4-coumaric acid, divided by the tritium/carbon-14 ratio in the reisolated intermediate, *trans*-cinnamic acid. Open bars, coupling factors in microsomes alone; filled bars, coupling factors in microsomes with 100 μL of the soluble PAL fraction added.

Data shown are the means ±SD of six (wild-type) or three (OX434 and C23) independent preparations.

We next studied the effects of transgenic modification of PAL activity on channeling in tobacco stem microsomes. *PAL*-overexpressing plants (OX434) had a two- to threefold higher specific activity of PAL, and PAL sense-suppressed plants (C23) a four- to sixfold lower specific activity of PAL in both soluble and microsomal fractions, when compared with wild-type plants (Figure 5A). Total microsomal activities were five to 10% of that detected in the soluble fraction, as was the case for wild-type tobacco plants. In microsomes from *PAL*-suppressed plants, coupling factors were in the same range as previously demonstrated for wild-type plants (Figure 5B). In contrast, the coupling factors for microsomes from *PAL*-overexpressing plants were reduced significantly to values of 1 to 2. These values are similar to those obtained on adding soluble PAL to wild-type microsomes. These results indicate that either higher PAL activity associated with the microsomes from *PAL*-overexpressing plants or the presence of a heterologous PAL species in the microsomes (see below) leads to a reduction in the extent of coupling between microsomal PAL and C4H.

Channeling between PAL and C4H in Microsomes from Tobacco Cell Suspension Cultures

If the changes in apparent metabolic compartmentalization of *trans*-cinnamic acid observed in in vivo labeling experiments indeed reflect changes in channeling rather than some in vivo labeling artifact, channeling should be demonstrable in microsomes from unelicited tobacco cell cultures but not in microsomes from elicited or *PAL*-overexpressing cultures. Such correlations between in vivo and in vitro labeling results would strengthen the validity and physiological relevance of the in vitro channeling assays.

The specific activity of soluble PAL in unelicited cell suspension cultures overexpressing bean *PAL2* was approximately sixfold higher than in wild-type cell suspension cultures, as shown by comparing time zero values in Figures 6A and 6C. The coupling factor between PAL and C4H in microsomes from unelicited wild-type cells (0 hr after elicitation) was ~12 (Figure 6B) and is similar to that observed in microsomes from stem tissue (Figure 5B). In agreement with the previous results obtained using stem tissue, microsomes from cell cultures overexpressing bean *PAL* had much reduced coupling factors, with a coupling factor below 1.0 and therefore indicative of no channeling (Figure 6D).

We next determined the effects of elicitation on the in vitro microsomal coupling of PAL and C4H in the wild-type and *PAL*-overexpressing cell suspension cultures. Treatment with crude yeast elicitor led to an approximately three- to fivefold increase in soluble PAL activity in wild-type cultures by 12 hr after elicitation (Figure 6A) but to a much smaller fold increase in the *PAL*-overexpressing cultures (Figure 6C). Microsomal PAL activity in elicited wild-type cells reached its maximum, an approximately fivefold increase, 9 hr after elicitation, as shown in Figure 6B. However, the coupling factors

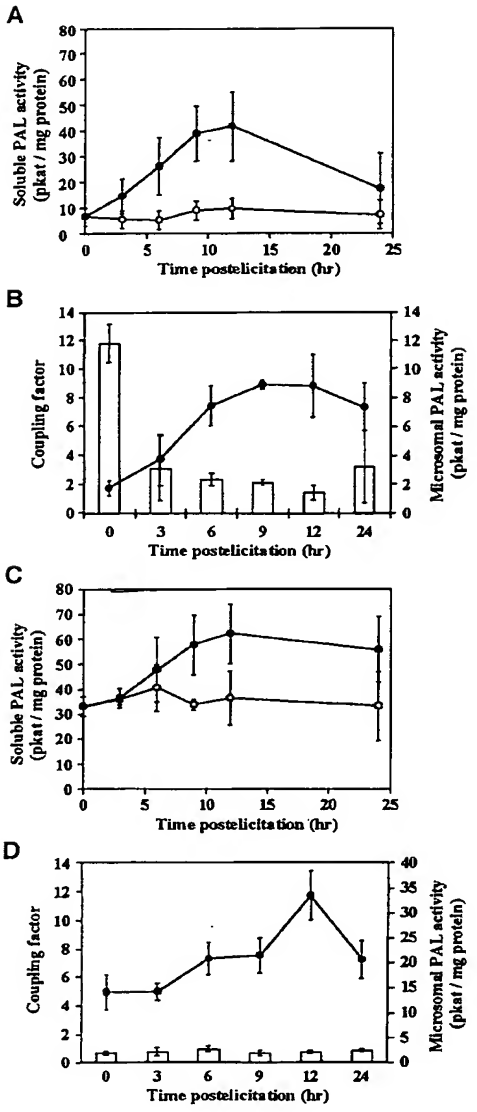

Figure 6. Specific Activities of PAL in the Soluble and Microsomal Protein Fractions, and Metabolic Channeling between PAL and C4H in Microsomes of Elicited Wild-Type and *PAL*-Overexpressing Tobacco Cell Suspension Cultures.

Isolation of microsomal proteins and determination of coupling factors were conducted as described in the legend to Figure 5. Wild-type and *PAL*-overexpressing cultures were treated with the yeast elicitor or an equal volume of water (unelicited) and then harvested at the times shown. pkat, picokatals.

(A) Soluble PAL activity in the unelicited (open circles) and elicited (filled circles) wild-type line.

(B) Microsomal PAL activity (filled circles) and coupling factors (open bars) in the elicited wild-type line.

(C) Soluble PAL activity in the unelicited (open circles) and elicited (filled circles) *PAL*-overexpressing lines.

(D) Microsomal PAL activity (filled circles) and coupling factors (open bars) in the elicited *PAL*-overexpressing lines.

Data shown are the means ±SD of four (wild-type) or three (*PAL*-overexpressing) independent experiments.

in microsomes from elicited wild-type cells decreased significantly by 3 hr after elicitation, to reach minimum values of ~1.4 at 12 hr after elicitation. These results demonstrate that the elicitation of PAL activity results in the perturbation of coupling between PAL and C4H. Elicitation did not strongly enhance microsomal PAL activity in *PAL*-overexpressing tobacco cells, and it had no effect on the coupling factors in microsomes from these cells, with the values being in the same range as in unelicited cells—near or below 1.0 (Figure 6D). Therefore, these data indicate a correlation between metabolic compartmentalization of *trans*-cinnamic acid in vivo (Figures 3 and 4) and coupling of the PAL and C4H reactions on microsomes in vitro.

The Nature of PAL Associated with Microsomal Membranes

The above-mentioned results suggest that PAL, or one or more specific forms of PAL, may be closely associated with C4H as an enzyme complex on microsomal membranes. Cytochrome P450s, such as C4H, are associated with the endoplasmic reticulum by way of a membrane anchor region at the N terminus (Chapple, 1998). The catalytic region of cytochrome P450 is in the cytoplasm. To obtain more information on the association of PAL with microsomal membranes, we fractionated tobacco stem homogenates by ultracentrifugation into soluble and microsomal fractions. Both fractions were assayed for PAL and C4H activity with or without a 20-min pretreatment with trypsin. The results in Table 3 indicate that as expected, no PAL activity could be detected in the soluble fraction after trypsin treatment. In contrast, 19% of the PAL activity in the microsomal fraction was retained. All microsomal C4H activity was destroyed by trypsin treatment. Because microsomal PAL activity is partially protected from the hydrolytic action of trypsin, a small fraction probably is located in the lumen of the endoplasmic reticulum or, alternatively, inside membrane vesicles formed during the preparation of microsomes. In this respect, the addition of the detergent Triton X-100 to the microsomal preparation before trypsin treatment resulted in an almost complete loss of microsomal PAL activity. The twofold increase in soluble PAL activity after the addition of detergent may indicate activation of the enzyme.

Tobacco PAL is encoded by two gene families, each of which contains two very closely related members. The two

Table 3. Effects of Trypsin on Soluble and Microsomal PAL Activity and Microsomal C4H Activity in Extracts from Wild-Type Tobacco Plants

	Total Activity (picokatals)[a]					
	Control		0.1 % Triton X-100		0.6 % Triton X-100	
Enzyme	−Trypsin	+Trypsin	−Trypsin	+Trypsin	−Trypsin	+Trypsin
Soluble PAL	392	0	696	0	668	0
Microsomal PAL	21	4	21	1	21	1
C4H	3	0	3	0	0	0

[a] Two grams of plant material was homogenized in 4 mL of extraction buffer and ultracentrifuged; the supernatant was assayed directly, and the pellet was resuspended in 250 μL of homogenization buffer. Fractions were incubated for 30 min at 4°C in the presence or absence of Triton X-100 before trypsin treatment (+Trypsin) or further incubation in the absence of trypsin (−Trypsin). Enzyme assays were started by the addition of substrate.

families are represented by single-copy *PAL* genes in the two progenitor species, *Nicotiana sylvestris* and *N. tomentosiformis* (Fukasawa-Akada et al., 1996). In this study, PAL1 refers to the product encoded by the *PAL* gene of family I, described by Fukasawa-Akada et al. (1996), and PAL2 refers to the product of the *PAL* gene from family II, as reported by Nagai et al. (1994). To determine whether specific forms of PAL are associated with tobacco microsomes, we raised antibodies against synthetic peptide sequences specific for tobacco PAL1, tobacco PAL2, and bean PAL2 (Howles et al., 1996). Soluble and microsomal proteins were isolated from wild-type, *PAL*-overexpressing (from the bean *PAL2* transgene), and *PAL*-suppressed tobacco stem tissues, subjected to SDS-PAGE, and probed with these antibodies. As shown in Figure 7A, antibodies generated against tobacco PAL1 cross-reacted with a protein band of just under 86 kD (the size of the native PAL subunit), which was present in both the soluble and microsomal protein fractions. By contrast, antibodies specific for tobacco PAL2 cross-reacted with a more diffuse protein band of similar size found only in the soluble protein fraction (Figure 7B). Note that the levels of tobacco PAL proteins were similar in wild-type, *PAL*-overexpressing, and *PAL*-suppressed plants, despite the different levels of PAL activity in these plants; this may be due to post-translational regulation.

Probing the protein gel blots with antibodies specific for bean PAL2 protein (Figure 7C) revealed that a very high amount of bean PAL2 protein is present in the soluble protein fraction, but a substantial amount of the heterologous gene product also is located in the microsomes of *PAL*-overexpressing plants. The ~50-kD band in the microsomal fraction cross-reacting with all three antibodies is most probably the result of nonspecific binding. Essentially identical results were obtained by protein gel blot analysis of soluble and microsomal fractions from cell suspension cultures of wild-type and *PAL*-overexpressing tobacco (data not shown). Furthermore, we could not detect any significant change in the subcellular localization of the various PAL forms after elicitation of the cell cultures (data not shown).

DISCUSSION

Relation of Metabolic Compartmentalization of *trans*-Cinnamic Acid in Vivo to Metabolic Channeling as Revealed by Coupling Assays in Vitro

As discussed by Srere (1987), objections have been raised to the idea that isotopic data can unequivocally indicate more than one pool of a particular intermediate. The major argument is that multiple pools arise due to heterogeneity of the cell population. De-differentiated tobacco cell suspension cultures are highly homogeneous with respect to cell type but somewhat heterogeneous with respect to cell size and degree of aggregation of small cell clusters. We cannot rule out totally the presence of different cell types containing different pools of intermediates that are differentially accessible to an endogenously formed or externally applied intermediate. However, this is unlikely because the isotopic labeling results are reproducible between independent cell culture batches, the isotope dilution experiments reveal at least two pools of *trans*-cinnamic acid but only a single pool of 4-coumaric acid, and more importantly, the effects on compartmentalization of transgenic or elicitor-mediated perturbation of the pathway can be reproduced by using in vitro channeling assays. The difference in isotope dilution behavior of *trans*-cinnamic acid compared with 4-coumaric acid potentially could result from uptake of *trans*-cinnamic acid into an inaccessible compartment, such as the cell wall, whereas all of the 4-coumaric acid may be taken into the cytoplasm. This appears not to be the case because if *trans*-cinnamic acid were taken up in this way, isotope dilution experiments with *PAL*-overexpressing transgenic cell cultures

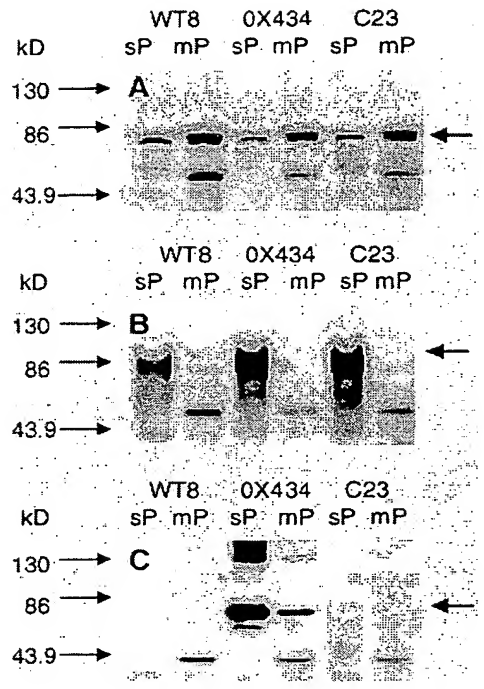

Figure 7. Protein Gel Blot Analysis of PAL Protein Levels in the Soluble and Microsomal Fractions from Wild-Type and Transgenic Tobacco Plants.

WT8 is a wild-type plant; OX434 is a *PAL*-overexpressing line; C23 is a *PAL*-suppressed line. Soluble PAL (sP) represents the PAL protein in the 130,000*g* supernatant, and microsomal PAL (mP) the PAL protein in the 130,000*g* pellet. Proteins (10 μg per lane) were separated by SDS-PAGE.

(A) Tobacco PAL1 protein detected using the anti-tobacco PAL1 antiserum.

(B) Tobacco PAL2 protein detected using the anti-tobacco PAL2 antiserum.

(C) Bean PAL2 protein detected using the anti-bean PAL2 antiserum.

Positions of the molecular mass markers are indicated at left in kilodaltons. The 80-kD PAL subunits are marked with arrows at right.

would artifactually reveal metabolic compartmentalization, which these do not.

This study provides a significant advance over previous work in this area by showing that the alteration of flux into the pathway by transgenic upregulation of the first enzyme, or coinduction of all the enzymes of the putative channel, leads to a loss of channeling, as revealed by both isotope dilution experiments in vivo and microsomal channeling assays in vitro. Thus, the in vitro assays appear to reflect the in vivo situation. This is an important observation in view of the often-cited criticism that in vitro enzyme complexes may not function at the presumed cellular pH and concentration conditions of the cell (Srere, 1987).

Note that the variation we observed in the product/precursor specific activity ratios determined in vivo is quite small, considering the potential biological variation between different cell culture batches, and the values for variation in the in vitro coupling factors are similar. In the latter case, the variation is probably a composite of biological variability and variation caused by sensitivity to disruption of the microsomal association between PAL and C4H. In this respect, it previously has been suggested that reversible inhibition of protein and nucleotide biosynthesis in gently sonicated yeast cells results from disruption of loosely associated enzyme complexes (Burns, 1964).

Previous studies with cucumber hypocotyls showed that hormonal or environmental stimuli could affect channeling between PAL and C4H in vitro. Thus, a coupling factor of ~5.0 was reduced to ~1.5 after exposure of green hypocotyls to ethylene, whereas it was increased to ~25 after irradiation with high-intensity UV light (Czichi and Kindl, 1977). Paradoxically, the latter treatment was reported to induce soluble PAL activity but not to induce microsomal PAL (Czichi and Kindl, 1977). No in vivo labeling data are reported in this study. In contrast, elicitation of tobacco cell cultures results in coordinated induction of both PAL (soluble and microsomal) and C4H activities (Howles et al., 1999) but in a loss of channeling between PAL and C4H, as assessed by both in vivo isotope dilution and in vitro microsomal channeling assays. Note that elicitation induces a different set of defense responses from those induced by UV irradiation (Hahlbrock and Scheel, 1989). The loss of channeling in *PAL*-overexpressing plants and cell cultures could result from two possible mechanisms. Quantitatively, the overexpression of PAL without a corresponding increase in C4H could lead to a spillover of *trans*-cinnamic acid. Alternatively, the qualitative difference in the PAL proteins in the *PAL*-overexpressing suspension cell cultures may reduce channeling if the bean PAL2 protein cannot correctly couple with tobacco C4H.

Association of PAL with Microsomal Membranes

Metabolic channeling may have more than one cellular function. In its simplest form, it might provide for rapid turnover of low concentrations of labile intermediates that have no other metabolic functions (Srere, 1987). However, the phenylpropanoid pathway presents a more complex case. PAL is encoded by small multigene families in all of the plants studied to date, including Arabidopsis (Wanner et al., 1995), and the pathway has several downstream branches leading to functionally distinct end products. It has long been proposed that different forms of PAL may be involved in the synthesis of different end products, primarily on the basis of differential product inhibition of different PAL forms (Alibert et al., 1972; Jones, 1984). Localization of one or more specific PAL isoforms on the surface of the endoplasmic reticulum would provide the necessary structural basis for assembling complexes in which different PAL forms could

channel metabolites into different pathways of phenylpropanoid metabolism.

Previous studies have used immunolocalization and biochemical fractionation techniques to show the association of PAL with endoplasmic reticulum membranes (Czichi and Kindl, 1975, 1977; Wagner and Hrazdina, 1984; Hrazdina and Wagner, 1985). Our results now demonstrate microsomal association of specific forms of PAL. Thus, tobacco PAL1 is found in both soluble and microsomal fractions, whereas tobacco PAL2 is not found in microsomes. PAL activity measured in microsomal preparations from tobacco stem tissues therefore reflects the localization of specific PAL forms on microsomal membranes rather than an artificial entrapment of PAL proteins into microsomal vesicles formed during the isolation process. This suggests that the molecular basis for the channeling of *trans*-cinnamic acid is the coupling of specific PAL forms with C4H located together on the microsomal membranes.

Our data do not suggest a mechanism for the apparent loss of channeling after elicitation. Protein gel blot analysis did not reveal any major difference in the localization of PAL forms after elicitation. The mechanism could therefore be subtle, perhaps involving specific post-translational modifications to PAL or C4H, or might simply reflect a change in the in vivo PAL/C4H activity ratio, with a resultant spillover of *trans*-cinnamic acid.

Implications of Metabolic Channeling for Phenylpropanoid Pathway Regulation

The addition of *trans*-cinnamic acid to bean cell cultures inhibits PAL at the transcriptional level and induces the synthesis of a proteinaceous inactivator of PAL (Bolwell et al., 1986, 1988). Furthermore, downregulation of C4H by antisense gene expression in transgenic tobacco leads to a corresponding decrease in PAL activity, suggesting that *trans*-cinnamic acid is sensed as a metabolic regulator of phenylpropanoid pathway flux in vivo (J. Blount and R.A. Dixon, unpublished results). Tobacco PAL is particularly sensitive to direct inhibition by *trans*-cinnamic acid in vitro (O'Neal and Keller, 1970). Tight coupling between PAL and C4H therefore could maintain, in the microsomal "compartment," a low *trans*-cinnamic acid pool that would avoid feedback inactivation of PAL, as suggested by Noe et al. (1980). Note, however, that the addition of 10^{-4} M *trans*-cinnamic acid in our isotope dilution experiments did not appear to inhibit flux through the PAL reaction in vivo, as assessed by incorporation of tritium from ^{3}H–L-Phe into the various phenylpropanoid compounds (data not shown).

PAL-overexpressing tobacco cells, in which channeling through the C4H reaction is no longer measurable, accumulate twofold higher levels of the potentially antifungal coumarin glycoside scopolin and the corresponding aglycone scopoletin (Ahl Goy et al., 1993; Gutierrez et al., 1995) than do wild-type cells. It is not possible to conclude that this results from a release from channeling of *trans*-cinnamic acid because of uncertainty as to the biosynthetic origin of scopoletin. The levels of scopoletin in tobacco cell cultures treated with a yeast elicitor are much lower than those in untreated cells, which could be due to degradation by induced peroxidases in elicited cells, as has been shown in other systems (Gutierrez et al., 1995; Breton et al., 1997; Edwards et al., 1997). A similar decline of constitutive phenolics associated with cell wall incorporation has been described for isoflavonoids in elicitor-treated cell suspension cultures of *Pueraria lobata* (Park et al., 1995), and we assume that the decline in the levels of most hydroxycinnamic acid derivatives in elicited tobacco cells is associated with such further metabolism rather than being a consequence of changes in metabolic channeling. In contrast, the benzoic acid derivatives pHBA, salicylic acid, vanillic acid, and the partially characterized vanillin derivative accumulated to the same level in elicited and unelicited tobacco cells. There appears to be independent regulation of the metabolic pathways involved in the biosynthesis of hydroxycinnamic acid derivatives, which include lignin precursors, and the biosynthesis of benzoic acid derivatives, which include salicylic acid (Figure 1).

Although the majority of phenylpropanoid compounds derived from the natural products of plants require both PAL and C4H for their synthesis, salicylic acid probably is derived directly from *trans*-cinnamic acid by chain shortening and ring hydroxylation (Lee et al., 1995). It is tempting to speculate that salicylic acid is synthesized via an uncoupled form of PAL, for example, nonmicrosomally associated PAL2 in tobacco. However, tobacco PAL1 could be equally involved in salicylic acid biosynthesis, because overexpression of bean PAL, which, like tobacco PAL1, is localized both cytoplasmically and microsomally in transgenic tobacco stem tissue, results in increased salicylic acid production and corresponding increases in disease resistance in intact tobacco plants (Felton et al., 1999).

Our results obtained in experiments with *PAL*-overexpressing tobacco cell cultures and elicited wild-type cultures do not support the idea that a loss of channeling leads directly to a higher accumulation of salicylic acid produced from unchanneled *trans*-cinnamic acid, because the levels of salicylic acid are the same as those in wild-type tobacco cells. However, a marked difference can be seen in the accumulation of the vanillin derivative, which is 10 times higher in *PAL*-overexpressing tobacco cells when compared with wild-type cells, although it is not significantly induced by elicitation. Currently, we cannot conclude whether the accumulation of this compound in *PAL*-overexpressing cells is due to a perturbation in *trans*-cinnamic acid channeling, because the biosynthetic pathway(s) involved in the formation of benzoic acid derivatives, such as vanillic acid and vanillin, still is to be unequivocally elucidated (Zenk, 1965; Funk and Brodelius, 1990; Yazaki et al., 1991; Schnitzler et al., 1992).

Two important predictions concerning phenylpropanoid pathway organization now can be tested in the model

system of tobacco. The first is that the differential subcellular localization of tobacco PAL1 and PAL2 has functional consequences related to metabolic channeling. This prediction can be addressed by studying the metabolic consequences of differentially downregulating expression of *PAL1* or *PAL2*. The second is that tobacco PAL1, but not PAL2, will be in close physical association with C4H. This prediction can be addressed by immunolocalization studies by using transgenic plants expressing epitope-tagged PAL and C4H species. These experiments are currently in progress.

METHODS

Plant Material

Tobacco (*Nicotiana tabacum* cv Xanthi-nc) plants were either wild type or transformed with the bean phenylalanine (Phe) ammonia–lyase *PAL2* gene (Elkind et al., 1990). The transformed plants displayed either increased PAL activity (Howles et al., 1996) or epigenetic gene silencing with reduced levels of activity. Used as controls were untransformed wild-type plants or plants from which the *PAL2* transgene had segregated. All plants were grown under greenhouse conditions (18°C at night and 27°C by day) and harvested just before flowering. Stem samples were taken from internodes seven to 11, counting from the top, frozen in liquid N_2, and ground in a tissue grinder.

Callus cultures were initiated from leaf discs of wild-type and transformed tobacco plants, as described previously (Bate et al., 1994). Liquid cultures were initiated and maintained in a modified Schenk and Hildebrandt medium, as described previously (Dixon et al., 1981), and subcultured every 7 to 10 days.

Five days after subculturing, dark-grown tobacco cell suspension cultures (75-mL batches) were treated with a yeast elicitor (Schumacher et al., 1987; 75 μg mL^{-1} glucose equivalents) and harvested at various times after elicitation. Control cells were treated with the same amount of distilled water.

Chemicals

^{3}H-L-2,3,4,5,6-Phe (124 Ci/mmol) and uniformly labeled L-^{14}C-Phe (453 mCi/mmol) were supplied by Amersham (Little Chalfont, United Kingdom). ^{14}C-*trans*-Cinnamic acid was synthesized enzymatically from uniformly labeled L-^{14}C-Phe by using PAL from *Rhodotorula glutinis* (14 units per mg of protein; Sigma) as described elsewhere (Edwards and Kessmann, 1992).

Precursor Dilution Experiments

Tobacco cell suspension cultures (75-mL batches) were incubated 4 days after subculturing with 7.5 μmol of ^{3}H-L-Phe (1 μCi/μmol) with or without unlabeled *trans*-cinnamic acid or 4-coumaric acid (0.75 or 7.5 μmol). After 24 hr, the cells were filtered through a nylon mesh and ground in liquid N_2. The soluble phenolics were extracted three times with 8 mL of ice-cold acetone at 4°C in the dark. The extracts were combined and concentrated to dryness under a stream of N_2, and the residue was dissolved in methanol (500 μL per gram fresh weight). Aliquots (20 μL) were separated by HPLC, as described below. Fractions of 500 μL were collected and counted in a liquid scintillation counter.

Enzymatic Hydrolysis of Phenolic Extracts

For the enzymatic hydrolysis of phenolic esters of caffeic acid, 4-coumaric acid, ferulic acid, and *trans*-cinnamic acid, 200 μL of the extracts was concentrated, dissolved in buffer (200 mM Tris-HCl, pH 8.0), and incubated overnight at 37°C with an esterase from rabbit liver (190 units; EC 3.1.1.1; Sigma). Phenolic glucosides of scopoletin, the vanillin derivative, *p*-hydroxybenzaldehyde (pHBA), and salicylic acid were hydrolyzed with almond β-glucosidase (100 units; EC 3.2.1.21; Sigma). Extracts then were processed for analysis of phenolic aglycones as described above.

Separation of Phenolics by Reverse Phase HPLC

Organic extracts from enzyme assays and plant or cell suspension phenolic fractions were applied to an ODS reverse phase HPLC column (5-mm particle size, 4.6 × 250 mm; Metachem Technologies, Inc., Torrance, CA) and eluted in 1% phosphoric acid with an increasing acetonitrile concentration gradient (0 to 5 min, 5% [v/v] acetonitrile; 5 to 10 min, 5 to 10% acetonitrile; 10 to 25 min, 10 to 17% acetonitrile; 25 to 30 min, 17 to 23% acetonitrile; 30 to 65 min, 23 to 50% acetonitrile; 65 to 74 min,100% acetonitrile; and 74 to 85 min, 5% acetonitrile) at a constant flow rate of 1 mL min^{-1}. UV absorbance was monitored with a photodiode array detector (Hewlett Packard, Waldbronn, Germany). Quantification of phenolics was based on calibration curves achieved with authentic standards (Sigma) at 270 and 330 nm.

Preparation of Membrane Fractions

Frozen (−70°C) and ground stem material or suspension cells (4 to 4.5 g fresh weight) were homogenized for 3 × 10 sec in 8 mL of a Tris-HCl buffer (200 mM Tris, pH 8.0, 400 mM sucrose, 1 mM EDTA, 40 mM sodium ascorbate, and 5 mM 2-mercaptoethanol) by using an Ultraturrax blender (Brinkmann Instruments, Inc., Westbury, NY). The homogenate was centrifuged (10,000*g* for 30 min) and filtered through a syringe filled with glass wool. The filtrate was ultracentrifuged (130,000*g* for 1 hr), the supernatant was decanted, and the pellet was blot dried. After resuspending the pellet in 2.5 mL of Pi buffer (200 mM potassium phosphate, pH 8.0, and 3 mM 2-mercaptoethanol) with a rubber spatula, the suspension was subjected to a second ultracentrifugation (130,000*g* for 1 hr). The supernatant was decanted, and the microsomal pellet was blot dried. The microsomes were resuspended carefully in 300 μL of assay buffer (200 mM potassium phosphate, pH 8.0, 6 mM $MgCl_2$, and 3 mM 2-mercaptoethanol). In all cases, 2-mercaptoethanol was added fresh to the buffers, and all steps were conducted on ice or at 4°C.

PAL and Cinnamic Acid 4-Hydroxylase Assays

Soluble PAL activity was determined in the ultracentrifugation supernatants (desalted on a PD-10 column equilibrated with 200 mM boric acid, pH 8.8, and 13 mM 2-mercaptoethanol) by using ^{14}C-L-Phe as a substrate, essentially as described by Legrand et al. (1976). For de-

termination of PAL activity in the microsomal fraction, 25 μL of the microsomal suspension was diluted with 25 μL of boric acid (200 mM, pH 8.8, containing 13 mM 2-mercaptoethanol) before adding the substrate. Cinnamic acid 4-hydroxylase (C4H) activity was determined in the microsomal fraction according to Edwards and Kessmann (1992). Protein concentrations were determined according to Bradford (1976), using BSA as a standard.

In Vitro Channeling Assays

Washed microsomes (200 μL) were preincubated with 245 μL of C4H assay buffer for 5 min, and the reactions were started with 500 nmol of ^{3}H-L-Phe (5 mCi/mmol), 30 nmol of ^{14}C-*trans*-cinnamic acid (2 mCi/mmol), and 1 mmol of NADPH in a total volume of 155 μL at 30°C. The reactions were stopped after 10 min with 50 μL of 6 N HCl.

Channeling assays in the presence of soluble PAL were conducted with 200 μL of the microsomal suspension, 100 μL of the soluble enzyme fraction, 145 μL of the C4H assay buffer, and the substrates, as described above. The assays were extracted with 3 × 700 μL of ethyl acetate, and the extracts were concentrated to dryness and dissolved in 30 μL methanol. Caffeic acid (1 μg) was added to the assays as an internal standard before extraction. Products were separated by reverse phase HPLC and monitored by UV absorbance at 270 nm, and the fractions (250 μL) were collected. The radioactivity in fractions containing 4-coumaric acid and *trans*-cinnamic acid was determined by liquid scintillation counting, by using the automatic quench compensation for tritium/carbon-14 dual label counting on an LS 1701 scintillation counter (Beckmann Instruments, Fullerton, CA).

Treatment of Plant Extracts with Trypsin

Soluble and microsomal enzyme fractions were prepared as described above, incubated with Triton X-100 (Sigma) (final concentration of 0.1 or 0.6%) or homogenization buffer (controls) for 30 min at 4°C, and then treated with 1 mg mL^{-1} trypsin (10,200 units per mg of protein; Sigma) for 20 min at 30°C. PAL and C4H enzyme assays were started by the addition of substrates, incubated for 20, 40, and 60 min, and stopped with HCl as described above.

Generation of Antibodies Specific for Tobacco and Bean PAL Forms

For raising antibodies specific to tobacco PAL proteins, the following amino acid sequences, derived from the available tobacco PAL sequences (Nagai et al., 1994; Pellegrini et al., 1994; Fukasawa-Akada et al., 1996), were used: VRDKSANG (positions 69 to 76 from tobacco PAL1) and VAQNGHQEMDFCVKV (positions 4 to 18 from tobacco PAL2). These sequences represent the few stretches that are unique between the different tobacco PAL forms. Synthetic peptides were coupled to the carrier protein keyhole limpet hemocyanin, and antibodies were raised in rabbits (Genosys Biotechnologies, Inc., The Woodlands, TX). Antibodies specific for bean PAL2 have been described previously (Howles et al., 1996).

Protein Gel Blot Analysis

Soluble and microsomal proteins (10 μg) were subjected to denaturing SDS-PAGE on precast 8 to 16% Tris-glycine gels (Novex, San Diego, CA) and transferred to nitrocellulose membranes (Trans-Blot; Bio-Rad). The membranes were blocked and probed with the primary antibody in 5% fat-free milk powder (Carnation, Glendale, CA) dissolved in 0.2% Tween 20 (Sigma) in Tris-buffered saline (TBST; Ausubel et al., 1994). A goat anti-rabbit IgG–horseradish peroxidase conjugate (Bio-Rad) was used as secondary antibody at a 1:10,000 dilution in TBST. Bands were visualized on a film by using a chemiluminescence assay (ECL; Amersham). Kaleidoscope prestained standards (Bio-Rad) were used as molecular weight markers.

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